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Sincerely,

Prof. Dr. Bektas TEPE, Ph.D.
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RESEARCH ARTICLE

OPEN ACCESS

Traditional medicinal plants used in the treatment of diabetes: Ethnobotanical and ethnopharmacological studies and mechanisms of action

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ABSTRACT

The use of medicinal plants for the prevention and treatment of several diseases, particularly diabetes, remains the remedy and the sustainable source for many diseases. This survey was conducted out in Bejaia province in the center of Algeria to invent the main plants used in folk medicine to treat diabetes mellitus, their availability in this region, and the mode of their use. This study was carried out in 2019 in several municipalities of the study area. Ethnobotanical information was obtained using a questionnaire through direct interviews with 323 people with diabetes. Among people with diabetes interviewed, 82% present type 2 diabetes, from which more than 60% of them use medicinal plants against 36.84% only in type 1 diabetics. Diabetes affected age groups differently; the age range most affected was 61-80 years (43.96%). A total of 43 plant species belonging to 25 families were identified and listed in this study. The most frequent species used by patients are *Artemisia herba-alba* (34.42%), *Olea europaea* (13.66%), and *Ajuga iva* (11.47%). The part of the plant used depends on the plant; the aerial part was the more used (40.9%), followed by leaves (25%) and fruits (13.63%). The other parts, such as seed, root, flower, bark, bulb, epicarp, and rhizome, were used with low frequencies. It was also interesting to indicate that decoction and infusion were the systematic preparation methods compared to others (maceration, cooking with food, and fresh). The present study clearly showed that phytotherapy is widely adopted by center Algerian society, and there is a huge diversity of medicinal plants used for the complementary treatment of diabetes. Moreover, this investigation provides researchers with important information that can be exploited to develop anti-diabetic remedies.

1. Introduction

Diabetes mellitus (DM) is a severe metabolic disease characterized by chronic hyperglycemia due to defects in insulin secretion, insulin action, or both (Arya et al., 2012). Currently, diabetes mellitus is considered one of the most common chronic diseases in nearly all countries, and its prevalence worldwide, particularly type 2 diabetes, is constantly increasing (IDF, 2013). In 1995, about 135 million people were affected by diabetes, and an increase of 300 million cases is estimated by 2025 (Vlad and Popa, 2012). More recently, some studies have estimated that by 2030, more than 552 million people will have diabetes, which represents about 9.9% of the world's population (Whiting et al., 2011).

The treatment of diabetes is based primarily on diet, physical exercise, and pharmacological agents. Regarding the therapeutic aspect, although several drugs are available for the treatment of diabetes, some antidiabetic drugs cause serious side effects, such as

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digestive, hepatic, and renal problems. Due to the adverse drug effects, patients are increasingly using medicinal plants as an alternative to prevent and treat diabetes. Thus, in recent decades, research has been focused on new antidiabetic molecules of plant origin with fewer toxic effects (Zhang et al., 2016). Many plant species are currently used worldwide to treat diabetes and are considered a major source of new antidiabetic agents. More than 1200 different plants have been described as a traditional diabetes treatment (Eddouks et al., 2007). Of all the plants tested *in vitro*, 80% are potentially antidiabetic, and some of them are at the origin of the development of new drugs, as in the case of metformin, which was developed from *Galega officinalis* L. (Bailey and Day, 2004). However, very little is known about the active compounds of antidiabetic plants, either their structures or actions, thus preventing them from being used in standard diabetes care (Coman et al., 2012).

Drug treatment is the main method followed by people with diabetes to fight against people with diabetes. In addition to pharmacological agents, many plants are used as a complementary treatment by a significant percentage of diabetic patients (Allali et al., 2008). To conserve and enhance the Algerian heritage in medicinal plants, some ethnobotanical studies are conducted in some regions, particularly in the south and west of the country (Allali et al., 2008; Rachid et al., 2012; Telli et al., 2016). The results obtained from these studies confirm that the Algerian population is highly attached to phytotherapy and traditional treatment. Whereas, very few ethnobotanical surveys have been carried out in the center of Algeria (Boudjelal et al., 2013), especially in Bejaia province, while this region has a very diverse vegetation cover with a great large number of plants used as folk remedies. Thus, the purpose of the present investigation was to determine the antidiabetic medicinal plants mostly used by the Bejaia population, the part and mode of use, and their effects.

Table 1. Questionnaire card survey

Information about patients		Information about diabetes		Information about plants used		Plant actions
Age	.....	Type of diabetes	.....	Name of plants used	.....	Good, normal or low
Sex	.....	Duration of diabetes	.....			therapeutic effect
Place of residence (city or companion)	.....	Drug used	.....	Used part(s)	.....	
		Complications	.....	Mode of use	.....	

2. Materials and methods

2.1. Geographical location

The study was conducted in Bejaia province (Wilaya), called Vgayet in the local language (Kabyle). This region is situated in the central north of Algeria between latitude 36°45′21″ N and longitude 5°05′03″ E, and between 0 to 1900m of altitude. It is limited by the Mediterranean sea from the North, Jijel province from the East, Setif and Bordj-Bou-Arreridj provinces from the south, and Tizi Ouzou and Bouira provinces from the west. Bejaia covers a total area of 3223 km² (52 municipalities), with an estimated population of about 950 000 inhabitants using Kabyle as the predominant language. The density of population is estimated at 295 inhabitants/km² and the majority of which occupy urban areas (more than 80%) (ASWB, 2015).

2.2. Relief and climate

The study area is located in plain Tellian Atlas; it is marked by the importance of the mountainous land, which occupies about 75% of the total region area, crossed by the valley of Soummam and the plains located near the coast. Mediterranean climate dominates in this region, but it varies from one area to another. The coastal zone and the valley of Soummam enjoy a rainy and mild climate in winter, dry and hot in summer. However, the climate of the mountain areas is characterized by a dry and hot summer and a rainy and cold winter (Skouri, 1994). The average annual temperature is around 18 °C, while the average annual rainfall is about 700 mm (ASWB, 2015).

2.3. Forest ecosystem and vegetation cover

With about 100 km of coast, extending from west to east and about 80 km of large from sea to the continental region (from North to south), the study area is characterized by an important vegetation cover. The forest area covers a total of 122500 ha, which represents 37.57% of the total area region (Bejaia), of which 58700 ha of covered forests (about 47.91% of the total forest area) and 63800 ha (52.08%) of maquis where thousands of plant species are growing. In Gouraya National Park only, with 20.8 km² of area (less than 1/100 of total area), about 526 plant species grow, including 123 medicinal species, rare species such as *Euphorbia dendroides* L., *Bupleurum plantagineum* L., and *Lithospermum rosmarinifolium* L. and other species not mentioned in the flora of Algeria namely *Cheiranthus cheiri* L. and *Cheilanthes acrostica* (Balb.) Tod. In the rest of the region, the plant species are not completely inventoried (ASWB, 2015).

The total agricultural area represents approximately 45% of the total area of the region (129448 ha). The arboriculture is marked by the predominance of the olive tree (52800 ha), followed by fig tree (10303 ha) and citrus tree (2010 ha) (ASWB, 2015).

2.4. Collection of information

An ethnobotanical study was conducted from June to October 2019 in several municipalities of the study area. Ethnobotanical information was obtained using a questionnaire by direct interview with 323 people with diabetes. The diabetic patients were chosen randomly in several municipalities of Bejaia province and interviewed directly face to face or by phone. The information collected from people with diabetes was divided into four parts: information concerning patients, information about diabetes, information related to plants used, and information about plant actions (Table 1).

2.5. Identification of medicinal plants used

Most of the plants used by people with diabetes were obtained from herbalists. Some plants do not grow in this region, so they are imported from other regions of the country or other countries. The growth of each plant in the study area and its common name have been confirmed by former herbalists and ancient inhabitants of this region, and their scientific names have been assigned for each plant based on various bibliographical references.

2.6. Data analysis

Microsoft Excel was used to calculate different statistical parameters and to draw graphics. The importance of each plant in the treatment of diabetes was assessed by the relative frequency of citation (RFC) calculated using the following formula (Tardío and Pardo-de-Santayana, 2008): RFC = FC/N, where "FC" is the number of people with diabetes who mention the use of the species also known as the Frequency Citation, and "N" is the total number of people with diabetes using plants (0 < RFC < 1).

3. Results and discussion

In the present investigation, 323 diabetic patients are chosen randomly, 178 men (55.10%) and 145 women (44.90%); more than 82% of patients present type 2 diabetes, and 29.72% present chronic complications (Table 2). All diabetic patients interviewed were under pharmacological treatment, but only 183 (56.65%) use antidiabetic medicinal plants, either regularly or rarely, and more than a third of them (34.97%) use more than one plant to cure diabetes.

In addition, this study shows that the use of medicinal plants is more apparent in type 2 diabetic population; more than 60% of type 2 diabetic patients use antidiabetic medicinal plants against 36.84% only in the type 1 diabetics (Table 2). It has also been observed that women use sensitively more medicinal plants than men, 59.31% and 54.49%, respectively. Moreover, our results show that the most affected age group is between 61 and 80 years with 43.96%; however, the antidiabetic medicinal plants are more used by the age group between 40 and 60 years with 61.90% (Table 3). This difference in the use of medicinal plants by diabetic groups is probably related to the type of diabetes and the severity of the disease which characterizes each group.

Table 2. Distribution of diabetics by sex, type of diabetes, and plant users

Sex	Number in percentage		Chronic complications	Diabetes type (%)		Diabetics using plants (%)	
	% of all diabetics	Using plants		T1D	T2D	T1D	T2D
Men	55.10	54.49	16.41	18.53	81.46	36.36	58.62
Women	44.90	59.31	13.31	16.55	83.44	37.5	63.63
All diabetics	-	56.65	29.72	17.64	82.35	36.84	60.90

Table 3. Distribution of diabetic plant users by categories

Age groups	20-40 years	41-60 years	61-80 years	> 80 years
% of diabetics	14.24	39	43.96	2.78
% of plant users	47.82	61.90	53.52	44.44

Concerning the use of antidiabetic plants by diabetic patients interviewed, a total of 43 medicinal plants belonging to 25 families were identified with a predominant use of Lamiaceae (18.60%) followed by Rosaceae (9.30%), Apiaceae, and Rutaceae (6.98%). The remaining plant families contribute by one or two species for each (2.33 or 4.65%).

The different plants used as antidiabetics can be divided into three origins, of which more than half (51.16%) grow in the wild, 39.53% are cultivated locally, and the rest (9.30%) are indigenous to other regions of Algeria or imported from other countries. The vernacular and binomial names of plants, used parts, preparation methods, number of citations (FC), and the relative frequency of citation of plant species (RFC) are illustrated in Table 4.

The most frequently used species by patients (used by over 5% of patients) were *Artemisia herba alba* Asso. (34.42%), *Olea europaea* L. (13.66%), *Ajuga iva* (L.) Schreb. (11.47%), *Rosmarinus officinalis* L. (8.19%), *Citrus limon* (L.) Osbeck. (7.10%), *Centaurium erythraea* Rafn. (6.01%), *Teucrium polium* L. (6.01%) and *Thymus serpyllum* L. (5.46%) (Figure 1).

The part of plants most commonly used by diabetic patients interviewed is the aerial part (Figure 2) with 40.9%, then leaf, fruit, and seed with 25%, 13.63%, and 6.81%, respectively; root, flower, bark, bulb, epicarp, and rhizome are also used but with a low percentage (2.27%).

About the preparation methods followed, the most adapted are decoction and infusion, respectively, with 79.06 and 58.13% (Figure 3). Other preparation methods such as maceration in water (11.62%) and cooking with food (4.65%) were also used. Sometimes, when it is necessary, the plant parts are taken fresh (11.62%).

We deduced in this part of the study that the preparation methods followed by diabetics are often in relation with plant part used; for example, the infusion is more used to extract active substances from soft parts such as leaves and flowers, while decoction is more used for hard parts such as roots, rhizome, and epicarp. However, fruits and vegetables are consumed fresh or cooked with other foods.

Although almost all diabetics do not have convincing scientific knowledge of the plant used, most of them are too satisfied by the antidiabetic effect obtained (Figure 4).

Many statistical studies recorded in several regions of the world reported that most diabetic patients are affected by type 2 diabetes with approximately 90% (WHO, 2016), and the most affected age group is situated between 60 and 80 years. In addition, the pharmacological treatment of diabetes is accompanied by traditional treatment in most societies of the world. However, the intensity and the manner of use of medicinal plants differ from one region to another and are always linked to the heritage of medicinal plants in societies and their attachment to traditional medicine (Jamshidi-Kia et al., 2018).

The percentage of people with diabetes using plants registered in this study is different from that reported in other regions of the country. For example, in the west of Algeria, the ethnobotanical study conducted by Allali et al. (2008) on 634 people with diabetes showed that about 62% of patients use medicinal plants. Also, in southern Algeria, the investigation realized by Telli et al. (2016) showed that among 289 diabetic patients interviewed, 60.90% use antidiabetic plants. However, only 28.30% use medicinal plants among 470 people with diabetes interviewed in southwestern and northwestern Algeria (Rachid et al., 2012). This difference may be

related to the degree of availability of plants (vegetal cover) and the degree of attachment to traditional medicine by societies.

This result is in accordance with those revealed by other Algerian scientists. The results of Rachid et al. (2012) showed that more than 36% of type 2 diabetic patients used medicinal plants but only 17.16% in type 1 diabetes cases. Similarly, Allali et al. (2008) have noticed during their ethnobotanical study that more than 66% of type 2 diabetic patients used plants against 33.8% only in the type 1 diabetic population.

Table 4. List of medicinal plants used for the treatment of diabetes mellitus in the Bejaia region

Family plant species	Arab name (Local name)	English name	Statue	Used part	Use method	FC	RFC
Asteraceae							
A. herba alba	Chih	White wormwood	Imp.	A. part	Dec.	63	0.34
C. cardunculus	Kharchouf (Thagga)	Cardoon	Cu	A. part	Cooked	2	0.01
Anacardiaceae							
Pistacia lentiscus L.	Edharou (Amadagh)	Lentisk	W	Leaves	Dec.	2	0.01
Apiaceae							
P. crispum	Bakdounes (Mâadnous)	Parsley	Cu	A. part	Dec./Inf.	3	0.02
C. sativum	Kasbara (Lkousvar)	Coriander	Cu	A. part	Dec./Inf.	1	0.005
A. graveolens	Elkarfes (Lekrafez)	Celery	Cu	A. part	Dec./Inf.	2	0.01
Brassicaceae							
Lepidium sativum	Elrechad (Guerninouche)	Garden cress	W	Seeds	Fresh	1	0.005
Cactaceae							
Opuntia ficus-indica	Attine achaouki (Akarmus)	Prickly pear cactus	Cu, W	A. part	Dec.	2	0.01
Convolvulaceae							
Convolvulus arvensis	Leblab (Merraz voukal)	Field bindweed	W	A. part	Dec./Inf.	1	0.005
Cucurbitaceae							
Citrullus colocynthis	Elhandhal (Rammane lkhechkheche)	Bitter apple	Imp.	Fruit	Mac.	1	0.005
C. pepo	Elkoussa (Corgitt)	Zucchini	Cu	Fruit	Fresh	2	0.01
Fabaceae							
Ceratonia siliqua	Elkharoub (Akharrouve)	Carob	W	Leaves	Dec.	1	0.005
P. vulgaris	Elfasoulia (Louvia)	Green beans	Cu	Fruit	Cooked	1	0.005
Gentianaceae							
C. erythraea	Elkantrioun assaghir (Qlilou)	Centaury	W	A. part	Dec./Inf./ Mac.	11	0.06
Lamiaceae							
A. iva	Chendgoura (Chkendoureth)	Bugleweed	W	A. part	Dec./Inf.	21	0.11
Marrubium vulgare	Elfrassioune (Marnuyeth)	White horehound	W	A. part	Dec./Inf.	5	0.027
R. officinalis	Iklil aldjabel (Azir, Amezir)	Rosemary	W	Leaves	Dec.	15	0.08
M. piperita	Naânaâ (Naânaâ)	Peppermint	Cu	A. part	Dec./Inf.	4	0.02
O. basilicum	Errayhane (Lehvaq)	Basil	Cu	A. part	Dec./Inf.	1	0.005
Salvia officinalis	Mrimia (Kheyatta, Swak ennebi)	Sage	W	A. part	Dec./Inf./Mac.	2	0.01
T. polium	Eldjaâda (Jaâtta)	Felty germander	W	A. part	Dec./Inf.	11	0.06
T. serpyllum	Zaâtar (Zaâtar)	Wild thyme	W	A. part	Dec./Inf.	10	0.05
Lauraceae							
C. cassia	El korfa (Elqurfa)	Chinese cassia	Imp.	Bark	Dec./Inf./Mac.	7	0.04
L. nobilis	Elghar (Rand)	Laurel	Imp.	Leaves	Dec./Inf.	3	0.02
Liliaceae							
Allium sativum	Athoum (Thicherth, thiskerth)	Garlic	Cu	Bulb	Dec./Inf./Fresh	2	0.01
Lythraceae							
Punica granatum	Rommane (Rammane)	Pomegranate	Cu	Epicarp, Leaves	Dec.	4	0.02
Moraceae							
Ficus carica	Attine (Thanqultt)	Fig tree	Cu	Leaves	Dec.	3	0.02
Oleacea							
O. europaea	Azzitoune (Azmeour)	Olive	Cu, W	Leaves	Dec./Fresh	25	0.14
Papaveraceae							
Papaver rhoeas	Chaquaeque annouamane (Jihvudh, Wahrir)	Red poppy	W	A. part	Dec./Inf.	1	0.005
Plantaginaceae							
Globularia alypum	Alainoune (Thasselgha)	Alypoglobe daisy	W	Leaves	Dec./Inf./Mac.	7	0.04
Ranunculaceae							
Nigella sativa	Alhaba assaouda (Sinoudj)	Black cumin	W, Imp.	Seeds	Dec.	5	0.03
Rhamnaceae							
Rhamnus alaternus	Annabaq (Amliless)	Privet	W	A. part	Dec./Inf.	3	0.02
Rosaceae							
Prunus dulcis	Ellaouz (Louz)	Almond	Cu, W	Fruit	Fresh	1	0.005
P. persica	Elkhaoukhe (Ikhoukhe)	Peachtree	Cu	Leaves	Dec./Inf.	5	0.03
Malus domestica	Attouffah (Teffah)	Apple	Cu	Fruit	Fresh	2	0.01
Rubus fruticosus	Attout alalliq (Inigel)	Wild brambles (Blackberry)	W	Leaves	Dec./Inf.	2	0.01
Rubiaceae							
Coffea canephora	Alboune (Lqahwa)	Coffee	Imp.	Seeds	Inf.	1	0.005
Rutaceae							
C. limon	Allaymoune (Lqares)	Lemon	Cu	Fruit	Dec./Inf./Fresh	13	0.07
Ruta graveolens	Assadhab (Awarmi)	Rue	W	Roots	Dec.	1	0.005
C. sinensis	Albortoqual (Tchina)	Orange	Cu	Flowers	Inf.	1	0.005
Theaceae							
Camellia sinensis	Achay (Tay)	Tea	Imp.	Leaves	Dec./Inf.	3	0.02
Urticaceae							
Urtica dioica	Alquarasse (Azeggduf)	Stinging nettle	W	A. part	Dec./Inf.	1	0.005
Zingiberaceae							
Z. officinale	Zindjabil (Zindjabil)	Ginger	Imp.	Rhizome	Dec./Inf.	4	0.02

A. Part: Aerial part, Dec.: Decoction, Inf.: Infusion, Mac.: Maceration, Cu: Cultivated, Imp.: Imported, W.: Wild, FC: Number of diabetics who mentioned the use of the species, RFC: relative frequency of citation (0 < RFC < 1)

This result may be due to the multifactorial nature of type 2 diabetes, where it is possible to use plant extracts as a complementary remedy to oral antidiabetic drugs. While hyperglycemia in type 1 diabetes is controlled only by taking insulin due to the absolute deficiency of this hormone in this case of diabetes.

The study realized by Allali et al. (2008) in western Algeria also showed that women use more medicinal plants than men but with different frequencies to ours, 70% of women and 30% of men. Also, in southwestern and northwestern Algeria, Rachid et al. (2012) have observed in their study that women used herbal medicine more frequently than men, with a percentage of 37.60 and 18.84, respectively. This could be related to the deep attachment of Algerian women to the traditional use of plants than men not only in the medical field but also in gastronomy and cosmetics.

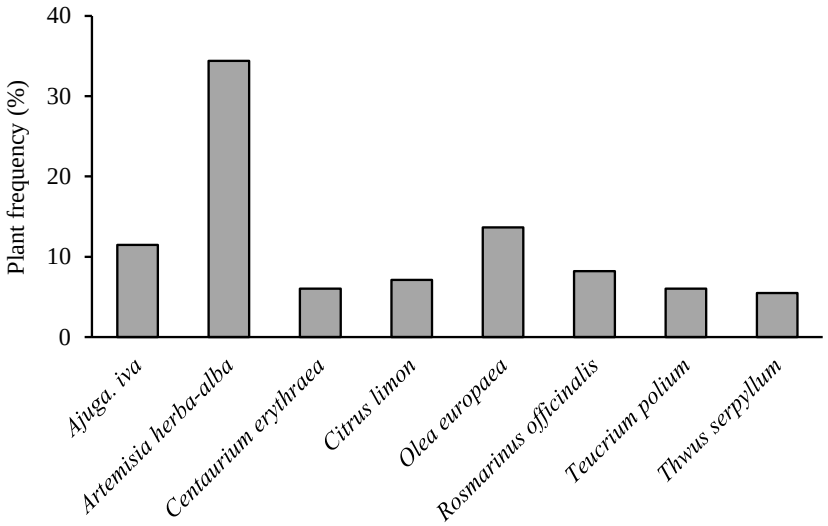

Figure 1. Frequency of more popular plants used by diabetic population

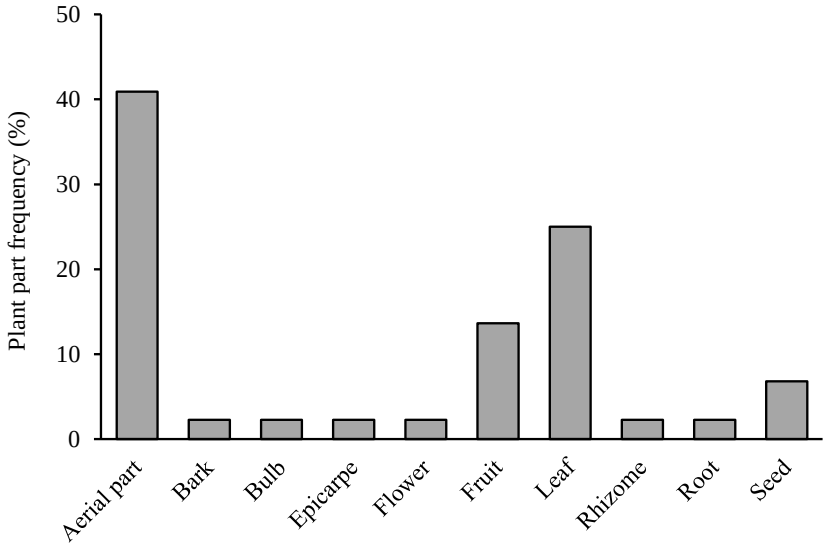

Figure 2. Frequency of plant parts used for the preparation of antidiabetic remedies

The preparation methods and the plant parts used are not based on scientific knowledge but rather on a traditional medicine heritage and social culture of people with diabetes (Rachid et al., 2012; Telli et al., 2016). They can also be recommended by herbalists' knowledge on the one hand and by the plant parts available during the year on the other hand.

The RFC of the plant used varied from 0.005 to 0.34 (Table 4). The highest value of RFC ranked was for *A. herba alba* (0.34), followed by *O. europaea* (0.14) and *A. iva* (0.11). The plants having the highest RFC are, in fact, predominantly used and commonly known by the local people. These may prove important for linking and evaluating research for future drug discovery and sustainable use of medicinal plants to treat diabetes. Some plants are widely cited in the bibliography as antidiabetic plants, and their antidiabetic effects have been demonstrated by several experimental studies. We have listed below the most cited plants in this survey, accompanied by some scientific studies in which they are cited.

A. herba alba (Asteraceae): It does not grow in the study area, but it is largely widespread in the highlands, the fresh semi-arid regions, and the steppes of Algeria (Jamshidi-Kia et al., 2018). It is the plant most used by diabetic using plants (34.42%). Its active compounds are extracted from the aerial part by decoction in all cases registered. In most cases, people with diabetes using this plant testifies to its satisfactory hypoglycemic action without any undesirable effect.

In fact, several experimental studies carried out in different regions of the world confirm the important antidiabetic effect of aerial part extracts of this plant (Awad et al., 2012; Boudjelal et al., 2015). Some studies demonstrated that extract of aerial part of *A. herba alba* has more anti-diabetic effect than root extract (Al-Khazraji et al., 1993).

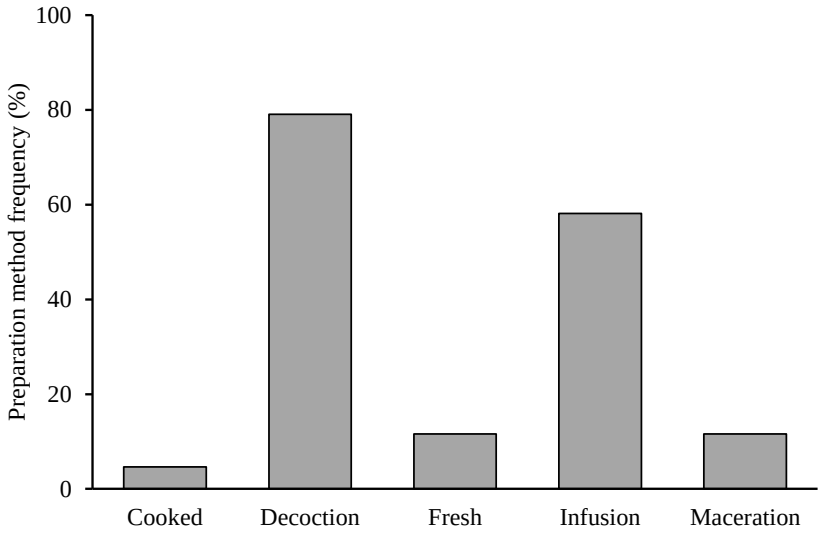

Figure 3. Preparation methods followed by diabetics to extract active substances

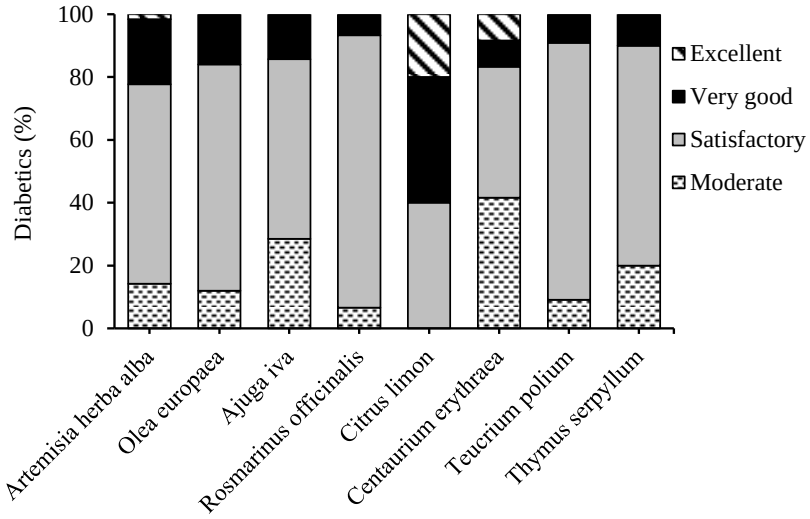

Figure 4. Testimony of diabetics about the anti-diabetic effect of plants used

Furthermore, many mechanisms of action have been suggested, such as the increase of peripheral glucose utilization (Taştekin et al., 2006), increasing insulin secretion (Awad et al., 2012), insulin-like action (Iriadam et al., 2006), and inhibition of the proximal tubular reabsorption for glucose in the kidneys (Mansi et al., 2007). The main components that may be responsible for these actions are flavonoids such as apigenin (flavones) (Awad et al., 2012) and volatile oil (Mahmoud et al., 2015).

O. europaea (Oleaceae): Largely abundant in the study area, about 3 929 418 trees with 63 varieties (ASWB, 2015). It is the second plant most used by people with diabetes (13.66%); the decoction of leaves is the method of use most adopted. However, some patients use fresh leaves by chewing.

Many experimental studies conducted in animals demonstrated that olive leaves extract possesses a potent antidiabetic action (Al-Attar and Alsalmi, 2019) without causing any toxicity (Clewell et al., 2016; Guex et al., 2018). The hypoglycemic effect has been attributed to phenolic compounds, especially: phenolic secoiridoids such as oleuropein (Annunziata et al., 2018), tannins (Wainstein et al., 2012), and flavonoids (Benhabyles et al., 2015).

The study conducted by Sato et al. (2007) showed that oleanolic acid (triterpene) extracted from olive leaves is an agonist for TGR5, a member of G-protein coupled receptor activated by bile acids and which implicated in glucose homeostasis.

Other studies showed that the potent antioxidant effect of oleuropein and other phenolic compounds of *O. europaea* could prevent diabetes and these complications by protecting body cells destruction (Al-Attar and Alsalmi, 2019; Büyükbalci and El, 2008; Guex et al., 2019; Jemai et al., 2009). Other mechanisms of action excreted by bioactive molecules of *O. europaea* leaves have been demonstrated, such as the increasing levels of liver insulin receptor substrate 1 (IRS1) and insulin receptor A (IRA) (Al-Attar and Alsalmi, 2019), the ameliorating effect of insulin secretion (Abdel-Kader et al., 2019), and by inhibition of α-amylase (Komaki et al., 2003).

A. iva (Lamiaceae): Abundant in arid and semi-arid areas of Algeria (Bendif et al., 2017). It also grows in the study region (Béjaia), but not in abundance; it is found especially in dry and stony ground. This plant is used by 11.47% of diabetic using plants, either by decoction or infusion of aerial part in most cases.

The antidiabetic effect of *A. iva* has been demonstrated by many experimental studies (Boudjelal et al., 2015; Wang et al., 2017) without any observable signs of toxicity (El Hilaly et al., 2004; Fettach et al., 2019; Tafesse et al., 2017). The main compounds of this plant responsible for the reduction of blood glucose are flavonoids, in particular, apigenin and naringenin (Boudjelal et al., 2015), triterpenoids, especially phytoecdysteroids (Wang et al., 2017), and phenolic acids (Khatteli et al., 2020). The *in vivo* study conducted by Wang et al. (2017) showed that phytoecdysteroids extracted from *A. iva* reduce blood glucose by regenerating pancreatic islets and upregulating hexokinase-I mRNA expression. Other studies showed that phenolic compounds of *A. iva* are potent inhibitors of α-glucosidase and α-amylase *in vivo* and *in vitro* (Fettach et al., 2019; Hsieh et al., 2014); therefore, they can significantly reduce postprandial blood sugar in diabetic patients.

R. officinalis (Lamiaceae): Very widespread in the study area, especially between 0 to 600 m of altitude. It grows wild in arid and dry regions of hills and low mountains (Fadili et al., 2015). Rosemary is used in Algerian folk medicine to treat hepatic diseases, eczema, hypertension (Boudjelal et al., 2013), colon ailments, stomachache, and hair loss (Senouci et al., 2019). In the present investigation, *R. officinalis* is used by 8.19% of people with diabetes using plants, and the decoction of leaves is the most method adopted. Several studies realized *in vivo* and *in vitro* showed the heightened hypoglycemic effect of *R. officinalis* extracts (Belmouhoub et al., 2018; Khalil et al., 2012; Labban et al., 2014). The bioactive molecules of *R. officinalis,* especially phenolic compounds such flavonoids and phenolic acids (Bakırel et al., 2008; Ibarra et al., 2011), can reduce hyperglycemia by various mechanisms, they can inhibit the α-glucosidase and α-amylase *in vivo* and *in vitro* (Belmouhoub et al., 2017; Koga et al., 2006), increase secretion of insulin by pancreatic cells (Ayaz, 2012; Bakırel et al., 2008) and regulate glucose metabolism in the liver (Tu et al., 2013).

C. limon (Rutaceae): This plant is cultivated in the study area, but the production of fruit is relatively low, about 6 730 tonnes for the 2014/2015 season (ASWB, 2015). In the present investigation, the fruit of *C. limon* is used by 7.10% of diabetic using plants. Most patients consume fresh fruit as a juice, while others use it in infusion or decoction. Patients using this fruit have testified that it has an excellent anti-diabetic effect; some among them have testified that they were completely cured of diabetes (Type 2 diabetes).

Recent publications have reported that *C. limon* flavonoids, particularly hesperidin, hesperetin, naringin, and naringenin, significantly decrease hyperglycemia by several action mechanisms (Alam et al., 2014; Lv et al., 2018). Akiyama et al. (2009) conducted an experimental study that showed that hesperidin significantly reduces blood glucose in diabetic rats by stimulating insulin secretion and regulating glucose metabolism in the liver. Naringin, another flavonoid of *C. limon,* can decrease blood glucose level in experimental animal models by decreasing glucose-6-phosphatase activity in the liver and by increasing hepatic glucokinase activity responsible for glycogen synthesis (Punithavathi et al., 2008), while naringenin reduces hyperglycemia by increasing muscle cell glucose uptake via adenosine monophosphate-activated protein kinase (AMPK) (Zygmunt et al., 2010).

C. erythraea (Gentianaceae): This plant grows wild in the study area, especially in the hills and in the wet and sunny pasture. In the present investigation, people with diabetes adopting this plant (6.01%) use its aerial part, either by decoction, infusion, or maceration, and they have testified that it has an excellent anti-diabetic effect; some among them have testified that they were completely cured of diabetes (Type 2 diabetes).

It has been demonstrated that aerial part extract of *C. erythraea* decreases hyperglycemia significantly in experimental animals; the hypoglycemic effect of the extract is attributed to flavonoids, mainly to flavonoid glycosides (Stefkov et al., 2011). The studies realized on diabetic rats showed that phenolic compounds of *C. erythraea* improve the structural and functional properties of pancreatic beta-cells by antioxidant regulatory mechanisms (Đorđević et al., 2019; Stefkov et al., 2014). On the other hand, the *in vitro* study carried out by Bouyahya et al. (2019) showed that essential oils of *C. erythraea* exhibit an important inhibition against α-amylase and α-glycosidase activity.

T. polium (Lamiaceae): It grows wild in the study area, especially in semi-arid soil, but not truly widespread; it predominates in the Tell region of the country, the highlands, and the Saharan Atlas (Quezel et al., 1962). Patients using this plant (6.01%) use its aerial part extracts obtained by infusion or decoction. The hypoglycemic effect of *T. polium* aerial part is attributed to several compounds, among them flavonoids, such as aglycon luteolin (Stefkov et al., 2014).

The antidiabetic properties of *T. polium* have been shown by various *in vitro* and *in vivo* studies (Ardestani et al., 2008; Dastjerdi et al., 2015; Esmaeili and Yazdanparast, 2004). Some compounds of *T. polium,* such as apigenin, can reduce hyperglycemia by stimulating pancreatic insulin secretion (Mirghazanfari et al., 2010). Other compounds, such as aromatic saturated and unsaturated fatty acids, and phenolic compounds, increase GLUT4 (glucose transporter 4) translocation to the cell membrane and enhance glucose uptake by cells skeletal muscle (Kadan et al., 2018). Furthermore, rutin and apigenin, two flavonoids isolated from *T. polium* protect pancreatic beta-cell against destruction and damage caused by oxidative stress (Esmaeili et al., 2009). However, many toxicological studies carried out on animal models have shown the toxic effect of *T. polium* extracts on the liver and kidneys (Abu Sitta et al., 2009; Al-Ashban et al., 2006; Ghasemi et al., 2019; Krache et al., 2017).

T. serpyllum (Lamiaceae): It grows wild in the study area, especially in mountains and high pastures. It is used by 5.46% of people with diabetes using plants. In all recorded cases, people with diabetes use only the aerial part either by decoction or infusion. The antidiabetic effect of this plant is attributed to several compounds such as flavonoids, alkaloids, tannins, and terpenoids (Mushtaq et

al., 2016). The *in vivo* study carried out by Alamgeer et al. (2014) showed that the aqueous extract of *T. serpyllum* significantly reduces hypoglycemia in glucose-fed mice. The mechanism of action and the toxic effect of *T. serpyllum* compounds are not truly elucidated, and there are very few studies in this way.

Although some plants constitute a part of the daily diet in Algeria, their relative frequency of citation in this investigation is very low (RFC < 0.05). These plants may be added daily to foods as condiments such as *Apium graveolens*, *Coriandrum sativum*, *Laurus nobilis*, *Ocimum basilicum*, *Petroselinum crispum*, and *Zingiber officinale* or consumed cooked as vegetables such as *Alium sativum*, *Cucurbita pepo*, *Cynara cardunculus*, *Phaseolus vulgaris* or consumed in herbal teas such as *Cinnamomum cassia* and *Mentha piperita*.

4. Conclusions

The present study clearly shows that phytotherapy is widely adopted by center Algerian society, and there is a huge diversity of medicinal plants used for the complementary treatment of diabetes. In addition, the majority of antidiabetic plants used are recognized by several scientific studies as antidiabetic and non-toxic plants. This confirms that the knowledge of societies about medicinal plants is a precious heritage, which must be preserved and transmitted to future generations.
Currently, it is accepted that the chronic complications of diabetes are caused mainly by oxidative stress generated by chronic hyperglycemia. In this context, certain hypoglycemic plants used by people with diabetes have a powerful antioxidant effect, which could considerably delay the onset of chronic complications when they are used as a complementary treatment.

During this study, some patients testified that they were completely cured of type 2 diabetes, and this is thanks to their daily use of medicinal plants, particularly patients using *C. limon* and *C. erythraea*.

Finally, this work constitutes a simple contribution of the Algerian heritage of medicinal plants in the treatment of diabetes; thorough pharmacological and phytochemical investigations are needed to reveal the real effect of these plants as well as their active components involved.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

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Messaoud Belmouhoub: Data curation, Writing - original draft, Conceptualization, Methodology
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Supplementary File

None.

References

Abdel-Kader, M.S., Soliman, G.A., Abdel-Rahman, R.F., Saeedan, A.S., Abd-Elsalam, R. M., Ogaly, H.A., 2019. Effect of olive leaves extract on the antidiabetic effect of glyburide for possible herb-drug interaction. *Saudi Pharmaceutical Journal*, 27(8), 1182-1195.

Abu Sitta, K.H., Shomah, M.S., Salhab, A.S., 2009. Hepatotoxicity of *Teucrium polium* L Tea: Supporting Evidence in Mice Models. *Australian Journal of Medical Herbalism*, 21(4), 106-109.

Akiyama, S., Katsumata, S.I., Suzuki, K., Ishimi, Y., Wu, J., Uehara, M., 2009. Dietary hesperidin exerts hypoglycemic and hypolipidemic effects in streptozotocin-induced marginal type 1 diabetic rats. *Journal of Clinical Biochemistry and Nutrition*, 46(1), 87-92.

Al-Ashban, R.M., Barrett, D.A., Shah, A.H., 2006. Effects of chronic treatment with ethanolic extract of *Teucrium polium* in mice. *Journal of Herbs, Spices & Medicinal Plants*, 11(4), 27-36.

Al-Attar, A.M., Alsalmi, F.A., 2019. Effect of *Olea europaea* leaves extract on streptozotocin induced diabetes in male albino rats. *Saudi Journal of Biological Sciences*, 26(1), 118-128.

Al-Khazraji, S.M., Al-Shamaony, L.A., Twaij, H.A., 1993. Hypoglycaemic effect of *Artemisia herba alba*. I. Effect of different parts and influence of the solvent on hypoglycaemic activity. *Journal of Ethnopharmacology*, 40(3), 163-166.

Alam, M.A., Subhan, N., Rahman, M.M., Uddin, S.J., Reza, H.M., Sarker, S.D., 2014. Effect of citrus flavonoids, naringin and naringenin, on metabolic syndrome and their mechanisms of action. *Advances in Nutrition*, 5(4), 404-417.

Alamgeer, M.N.M., Rashid, M., Malik, M.N.H., Ahmad, T., Khan, A.Q., Javed, I., 2014. Evaluation of Hypoglycemic activity of *Thymus serpyllum* Linn in glucose treated mice. *International Journal of Basic Medical Sciences and Pharmacy (IJBMSP)*, 3(2).

Allali, H., Benmehdi, H., Dib, M.A., Tabti, B., Ghalem, S., Benabadji, N., 2008. Phytotherapy of diabetes in west Algeria. *Asian Journal of Chemistry*, 20(4), 2701-2710.

Annunziata, G., Maisto, M., Schisano, C., Barrea, L., Ciampaglia, R., Novellinoet, E., 2018. Oleuropein as a novel anti-diabetic nutraceutical. An overview. *Archives of Diabetes & Obesity*, 1(3), 54-58.

Ardestani, A., Yazdanparast, R., Jamshidi, S.H., 2008. Therapeutic effects of *Teucrium polium* extract on oxidative stress in pancreas of streptozotocin-induced diabetic rats. *Journal of Medicinal Food*, 11(3), 525-532.

Arya, A., Looi, C.Y., Cheah, S.C., Mustafa, M.R., Mohd, M.A., 2012. Anti-diabetic effects of *Centratherum anthelminticum* seeds methanolic fraction on pancreatic cells, β-TC6 and its alleviating role in type 2 diabetic rats. *Journal of Ethnopharmacology*, 144(1), 22-32.

ASWB, 2015. Annuaire Statistique de la Wilaya de Bejaia. http://www.univ-bejaia.dz/doc/Annuaire%20Statistique%202015 (accessed November 07, 2021).

Awad, N.E., Seida, A.A., Shaffie, Z.E., El-Aziz, A.M., Awad, N.E., 2012. Hypoglycemic activity of *Artemisia herba-alba* (Asso.) used in Egyptian traditional medicine as hypoglycemic remedy. *Journal of Applied Pharmaceutical Science*, 2, 30-39.

Ayaz, N.O., 2012. Antidiabetic and renoprotective effects of water extract of *Rosmarinus officinalis* in streptozotocin-induced diabetic rat. *African Journal of Pharmacy and Pharmacology*, 6(37), 2664-2669.

Bailey, C.J., Day, C., 2004. Metformin: its botanical background. *Practical Diabetes International*, 21(3), 115-117.

Bakırel, T., Bakırel, U., Keleş, O.Ü., Ülgen, S.G., Yardibi, H., 2008. *In vivo* assessment of antidiabetic and antioxidant activities of rosemary (*Rosmarinus officinalis*) in alloxan-diabetic rabbits. *Journal of Ethnopharmacology*, 116(1), 64-73.

Belmouhoub, M., Bribi, N., Iguer-ouada, M., 2017. Alpha-glucosidase inhibition and antihyperglycemic activity of flavonoids rich fractions of *Rosmarinus officinalis* in normal and streptozotocin diabetic mice. *Oriental Pharmacy and Experimental Medicine*, 17(1), 29-39.

Belmouhoub, M., Chebout, I., Iguer-Ouada, M., 2018. Antidiabetic and anti-hypercholesterolemic effects of flavonoid-rich fractions of *Rosmarinus officinalis* in streptozotocin-induced diabetes in mice. *Phytotherapie*, 16(4), 204-210.

Bendif, H., Lazali, M., Harir, M., Miara, M.D., Boudjeniba, M., Venskutonis, P.R., 2017. Biological screening of *Ajuga iva* extracts obtained by supercritical carbon dioxide and pressurized liquid extraction. *Journal of Medicinal Botany*, 1, 33-41.

Benhabyles, N., Arab, K., Bouchenak, O., Baz, A., 2015. Phytochemical screening, hypoglycemic and antihyperglycemic effect of flavonoids from the leaves of Algerian

Olea europaea L. in normal and alloxan-induced diabetic rats. *International Journal of Pharmacology*, 11(5), 477-483.
Boudjelal, A., Henchiri, C., Sari, M., Sarri, D., Hendel, N., Benkhaled, A., Ruberto, G., 2013. Herbalists and wild medicinal plants in M'Sila (North Algeria): An ethnopharmacology survey. *Journal of Ethnopharmacology*, 148(2), 395-402.
Boudjelal, A., Siracusa, L., Henchiri, C., Sarri, M., Abderrahim, B., Baali, F., Ruberto, G., 2015. Antidiabetic effects of aqueous infusions of *Artemisia herba-alba* and *Ajuga iva* in alloxan-induced diabetic rats. *Planta Medica*, 81(09), 696-704.
Bouyahya, A., Belmehdi, O., El Jemli, M., Marmouzi, I., Bourais, I., Abrini, J., Bakri, Y., 2019. Chemical variability of *Centaurium erythraea* essential oils at three developmental stages and investigation of their *in vitro* antioxidant, antidiabetic, dermatoprotective and antibacterial activities. *Industrial Crops and Products*, 132, 111-117.
Büyükbalci, A., El, S.N., 2008. Determination of in vitro antidiabetic effects, antioxidant activities and phenol contents of some herbal teas. *Plant Foods for Human Nutrition*, 63(1), 27-33.
Clewell, A.E., Béres, E., Vértesi, A., Glávits, R., Hirka, G., Endres, J.R., Szakonyiné, I.P., 2016. A comprehensive toxicological safety assessment of an extract of *Olea europaea* L. leaves (bonolive™). *International Journal of Toxicology*, 35(2), 208-221.
Coman, C., Rugina, O.D., Socaciu, C., 2012. Plants and natural compounds with antidiabetic action. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 40(1), 314-325.
Dastjerdi, Z.M., Namjoyan, F., Azemi, M.E., 2015. Alpha amylase inhibition activity of some plants extract of *Teucrium* species. *European Journal of Biological Sciences*, 7(1), 26-31.
Đorđević, M., Grdović, N., Mihailović, M., Jovanović, J.A., Uskoković, A., Rajić, J., Dinić, S., 2019. *Centaurium erythraea* extract improves survival and functionality of pancreatic beta-cells in diabetes through multiple routes of action. *Journal of Ethnopharmacology*, 242, 112043.
Eddouks, M., Ouahidi, M.L., Farid, O., Moufid, A., Khalidi, A., Lemhadri, A., 2007. L'utilisation des plantes médicinales dans le traitement du diabète au Maroc. *Phytothérapie*, 5(4), 194-203.
El Hilaly, J., Israili, Z.H., Lyoussi, B., 2004. Acute and chronic toxicological studies of *Ajuga iva* in experimental animals. *Journal of Ethnopharmacology*, 91(1), 43-50.
Esmaeili, M.A., Yazdanparast, R., 2004. Hypoglycaemic effect of *Teucrium polium*: studies with rat pancreatic islets. *Journal of Ethnopharmacology*, 95(1), 27-30.
Esmaeili, M.A., Zohari, F., Sadeghi, H., 2009. Antioxidant and protective effects of major flavonoids from *Teucrium polium* on β-cell destruction in a model of streptozotocin-induced diabetes. *Planta Medica*, 75(13), 1418-1420.
Fadili, K., Amalich, S., N'dedianhoua, S.K., Bouachrine, M., Mahjoubi, M., El Hilali, F., Zair, T., 2015. Polyphenols content and antioxidant activity of two species from Moroccan High Atlas: *Rosmarinus officinalis* and *Thymus satureioides*. *International Journal of Innovation and Scientific Research*, 4(17), 24-33.
Fettach, S., Mrabti, H.N., Sayah, K., Bouyahya, A., Salhi, N., Cherrah, Y., El Abbes, F.M., 2019. Phenolic content, acute toxicity of *Ajuga iva* extracts and assessment of their antioxidant and carbohydrate digestive enzyme inhibitory effects. *South African Journal of Botany*, 125, 381-385.
Ghasemi, T., Keshavarz, M., Parviz, M., 2019. Acute hepatorenal dose dependent toxicity of *Teucrium polium* hydro alcoholic extract in rat. *International Journal of Pediatrics*, 7(9), 10099-10107.
Guex, C.G., Reginato, F.Z., de Jesus, P.R., Brondani, J.C., Lopes, G.H.H., de Freitas Bauermann, L., 2019. Antidiabetic effects of *Olea europaea* L. leaves in diabetic rats induced by high-fat diet and low-dose streptozotocin. *Journal of Ethnopharmacology*, 235, 1-7.
Guex, C.G., Reginato, F.Z., Figueredo, K.C., da Silva, A.R.H., Pires, F.B., da Silva Jesus, R., de Freitas Bauermann, L., 2018. Safety assessment of ethanolic extract of *Olea europaea* L. leaves after acute and subacute administration to Wistar rats. *Regulatory Toxicology and Pharmacology*, 95, 395-399.
Hsieh, C.W., Cheng, J.Y., Wang, T.H., Wang, H.J., Ho, W.J., 2014. Hypoglycaemic effects of *Ajuga* extract *in vitro* and *in vivo*. *Journal of Functional Foods*, 6, 224-230.
Ibarra, A., Cases, J., Roller, M., Chiralt-Boix, A., Coussaert, A., Ripoll, C., 2011. Carnosic acid-rich rosemary (*Rosmarinus officinalis* L.) leaf extract limits weight gain and improves cholesterol levels and glycaemia in mice on a high-fat diet. *British Journal of Nutrition*, 106(8), 1182-1189.
IDF, 2013. International Diabetes Federation. https://www.idf.org/ (accessed December 28, 2021).
Iriadam, M., Musa, D., Gumushan, H., Baba, F., 2006. Effects of two Turkish medicinal plants *Artemisia herba-alba* and *Teucrium polium* on blood glucose levels and other biochemical parameters in rabbits. *Journal of Molecular Cell Biology*, 5(1), 19-24.
Jamshidi-Kia, F., Lorigooini, Z., Amini-Khoei, H., 2018. Medicinal plants: Past history and future perspective. *Journal of Herbmed Pharmacology*, 7(1), 1-7.
Jemai, H., El Feki, A., Sayadi, S., 2009. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. *Journal of Agricultural and Food Chemistry*, 57(19), 8798-8804.
Kadan, S., Sasson, Y., Abu-Reziq, R., Saad, B., Benvalid, S., Linn, T., Zaid, H., 2018. *Teucrium polium* extracts stimulate GLUT4 translocation to the plasma membrane in L6 muscle cells. *Advancement in Medicinal Plant Research*, 6(1), 1-8.
Khalil, O.A., Ramadan, K.S., Danial, E.N., Alnahdi, H.S., Ayaz, N.O., 2012. Antidiabetic activity of *Rosmarinus officinalis* and its relationship with the antioxidant property. *African Journal of Pharmacy and Pharmacology*, 6(14), 1031-1036.
Khatteli, A., Benabderrahim, M.A., Triki, T., Guasmi, F., 2020. Aroma volatiles, phenolic profile and hypoglycaemic activity of *Ajuga iva* L. *Food Bioscience*, 36, 100578.
Koga, K., Shibata, H., Yoshino, K., Nomoto, K., 2006. Effects of 50% Ethanol Extract from Rosemary (*Rosmarinus officinalis*) on α-Glucosidase Inhibitory Activity and the Elevation of Plasma Glucose Level in Rats, and Its Active Compound. *Journal of Food Science*, 71(7), S507-S512.
Komaki, E., Yamaguchi, S., Maru, I., Kinoshita, M., Kakehi, K., Ohta, Y., Tsukada, Y., 2003. Identification of anti-α-amylase components from olive leaf extracts. *Food Science and Technology Research*, 9(1), 35-39.
Krache, I., Boussoualim, N., Ouhida, S., Amraoui, N., Baghiani, A., Arrar, L., 2017. Acute and chronic effects of methanolic extract of *Teucrium polium* on blood parameters and histopathology of liver and kidney in female rats. *Asian Journal of Research in Medical and Pharmaceutical Sciences*, 2, 1-11.
Labban, L., Mustafa, U.E.S., Ibrahim, Y.M., 2014. The effects of rosemary (*Rosmarinus officinalis*) leaves powder on glucose level, lipid profile and lipid perodoxation. *International Journal of Clinical Medicine*, 2014.
Lv, J., Cao, L., Li, M., Zhang, R., Bai, F., Wei, P., 2018. Effect of hydroalcohol extract of lemon (*Citrus limon*) peel on a rat model of type 2 diabetes. *Tropical Journal of Pharmaceutical Research*, 17(7), 1367-1372.
Mahmoud, A.M., Ahmed, O.M., Ashour, M.B., Abdel-Moneim, A., 2015. *In vivo* and *in vitro* antidiabetic effects of citrus flavonoids; a study on the mechanism of action. *International Journal of Diabetes in Developing Countries*, 35(3), 250-263.
Mansi, K., Amneh, M., Nasr, H., 2007. The hypolipidemic effects of *Artemisia sieberi* (*A. herba-alba*) in alloxan induced diabetic rats. *International Journal of Pharmacology*, 3(6), 487-491.
Mirghazanfari, S.M., Keshavarz, M., Nabavizadeh, F., Soltani, N., Kamalinejad, M., 2010. The effect of "*Teucrium polium* L." extracts on insulin release from *in situ* isolated perfused rat pancreas in a newly modified isolation method: the role of Ca^{2+} and K^{+} Channels. *Iranian Biomedical Journal*, 14(4), 178.
Mushtaq, M.N., Bashir, S., Ullah, I., Karim, S., Rashid, M., Hayat Malik, M.N., 2016. Comparative hypoglycemic activity of different fractions of *Thymus serpyllum* L. in alloxan induced diabetic rabbits. *Pakistan Journal of Pharmaceutical Sciences*, 29(5), 1483–1488.
Punithavathi, V.R., Anuthama, R., Prince, P.S.M., 2008. Combined treatment with naringin and vitamin C ameliorates streptozotocin-induced diabetes in male Wistar rats. *Journal of Applied Toxicology: An International Journal*, 28(6), 806-813.
Quezel, P., Santa, S., Schotter, O., 1962. Nouvelle flore de l'Algerie et des regions desertiques meridionales. Volume 2. Editions du Centre National de la Recherche Scientifique, Paris.
Rachid, A., Rabah, D., Farid, L., Zohra, S.F., Houcine, B., Nacéra, B., 2012) Ethnopharmacological survey of medicinal plants used in the traditional treatment of diabetes mellitus in the North Western and South Western Algeria. *Journal of Medicinal Plants Research*, 6(10), 2041-2050.
Sato, H., Genet, C., Strehle, A., Thomas, C., Lobstein, A., Wagner, A., Saladin, R., 2007. Anti-hyperglycemic activity of a TGR5 agonist isolated from *Olea europaea*. *Biochemical and Biophysical Research Communications*, 362(4), 793-798.
Senouci, F., Ababou, A., Chouieb, M., 2019. Ethnobotanical survey of the medicinal plants used in the Southern Mediterranean. Case study: the region of Bissa (Northeastern Dahra Mountains, Algeria). *Pharmacognosy Journal*, 11(4), 647-659.
Skouri, M., 1994. Les ressources physiques de la région méditerranéenne, In: Dupuy, B. (Ed.) Equilibre alimentaire, agriculture et environnement en Méditerranée. Ciheam, Montpellier, pp. 15–30.
Stefkov, G., Kulevanova, S., Miova, B., Dinevska-Kjovkarovska, S., Mølgaard, P., Jäger, A. K., Josefsen, K., 2011. Effects of *Teucrium polium* spp. *capitatum* flavonoids on the lipid and carbohydrate metabolism in rats. *Pharmaceutical Biology*, 49(9), 885-892.
Stefkov, G., Miova, B., Dinevska-Kjovkarovska, S., Stanoeva, J.P., Stefova, M., Petrusevska, G., Kulevanova, S., 2014. Chemical characterization of *Centaurium erythrea* L. and its effects on carbohydrate and lipid metabolism in experimental diabetes. *Journal of Ethnopharmacology*, 152(1), 71-77.
Tafesse, T.B., Hymete, A., Mekonnen, Y., Tadesse, M., 2017. Antidiabetic activity and phytochemical screening of extracts of the leaves of *Ajuga remota* Benth on alloxan-induced diabetic mice. *BMC Complementary and Alternative Medicine*, 17(1), 1-9.
Tardío, J., Pardo-de-Santayana, M., 2008. Cultural importance indices: a comparative analysis based on the useful wild plants of Southern Cantabria (Northern Spain) 1. *Economic Botany*, 62(1), 24-39.
Taştekin, D., Atasever, M., Adigüzel, G., Keleş, M., Taştekin, A., 2006. Hypoglycaemic effect of *Artemisia herba-alba* in experimental hyperglycaemic rats. *Bulletin of the Veterinary Institute in Pulawy*, 50, 235-238.
Telli, A., Esnault, M.A., Khelil, A.O.E.H., 2016. An ethnopharmacological survey of plants used in traditional diabetes treatment in south-eastern Algeria (Ouargla province). *Journal of Arid Environments*, 127, 82-92.
Tu, Z., Moss-Pierce, T., Ford, P., Jiang, T.A., 2013. Rosemary (*Rosmarinus officinalis* L.) extract regulates glucose and lipid metabolism by activating AMPK and PPAR pathways in HepG2 cells. *Journal of Agricultural and Food Chemistry*, 61(11), 2803-2810.
Vlad, I., Popa, A.R., 2012. Epidemiology of diabetes mellitus: a current review. *Romanian Journal of Diabetes Nutrition and Metabolic Diseases*, 19, 433–440.
Wainstein, J., Ganz, T., Boaz, M., Bar Dayan, Y., Dolev, E., Kerem, Z., Madar, Z., 2012. Olive leaf extract as a hypoglycemic agent in both human diabetic subjects and in rats. *Journal of Medicinal Food*, 15(7), 605-610.
Wang, J.J., Jin, H., Zheng, S.L., Xia, P., Cai, Y., Ni, X.J., 2017. Phytoecdysteroids from *Ajuga iva* act as potential antidiabetic agent against alloxan-induced diabetic male albino rats. *Biomedicine & Pharmacotherapy*, 96, 480-488.

Whiting, D.R., Guariguata, L., Weil, C., Shaw, J., 2011. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Research and Clinical Practice*, 94(3), 311-321.

WHO, 2016. World Health Organization. Global Report on Diabetes. Geneva. http://apps.who.int/iris/bitstream/10665/204871/1/9789241565257_eng.pdf. (accessed October 12, 2021).

Zhang, Y., Wu, L., Ma, Z., Cheng, J., Liu, J., 2016. Anti-diabetic, anti-oxidant and anti-hyperlipidemic activities of flavonoids from corn silk on STZ-induced diabetic mice. *Molecules*, 21(1), E7.

Zygmunt, K., Faubert, B., MacNeil, J., Tsiani, E., 2010. Naringenin, a citrus flavonoid, increases muscle cell glucose uptake via AMPK. *Biochemical and Biophysical Research Communications*, 398(2), 178-183.

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RESEARCH ARTICLE

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Extraction, characterization, and evaluation of the functionality of fixed oil low-quality coffee beans for use as pharmaceutical ingredients

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ABSTRACT

In order to offer a viable destination for green coffee beans classified as non-beverage type, this work aimed to extract and characterize the fixed oil from these beans and perform a preliminary evaluation of its functionality as a pharmaceutical ingredient. The extraction yield obtained was 3.70 ± 1.29% (w/w). The oil present in its composition high levels of fatty acids with emulsifying and emollient properties, palmitic acid (47.76%) and linoleic acid (32.98%); and compounds with antioxidant functional properties, tocopherols (788.71 ± 56.08 mg/kg) and phenolic compounds (3312.40 ± 14.62 mg/kg). This oil showed antioxidant activity against the free radical 2,2-diphenyl-1-picryl hydrazil at all tested concentrations, reaching 50% inhibition at the concentration of 0.59 mg/ml and 90% at 0.96 mg/ml. The preliminary evaluation of the physical stability of the creams showed that, when incorporated into formulations, this oil has the potential to be used as a substitute for the synthetic ingredients liquid petrolatum, decyl oleate, and butylated hydroxytoluene.

1. Introduction

Brazil is the largest producer and exporter of coffee globally, and the species of greatest economic importance are *Coffea arabica* L. and *C. canephora* P., popularly known as arabica and conilon, respectively. In the 2020 harvest, production reached a total harvested volume of 63.08 million benefited bags, equivalent to approximately 3.8 million tons, with 77.3% corresponding to arabica coffee and 22.7% to conilon (Brasil, 2020a).

Regarding the production of Brazilian coffee, problems with the low quality of the beans have been observed. It is estimated that 12 million bags of coffee per year produced in Brazil do not meet the quality standards established by Normative Instruction 08/2003 of the Ministério da Agricultura Pecuária e Abastecimento-MAPA (Brasil, 2003; Brasil, 2020b; Kalschne et al., 2018). Defects and impurities can be due to genetic and physiological causes, problems in nutrition, attacks by pests and diseases, climatic conditions, in addition to external factors such as poor regulation of the processing machine and poor drying and storage structure, among others (Hameed et al., 2018; Poltronieri and Rossi, 2016).

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In order to be marketed, these low-quality coffees must go through the process of grain reclassification, carried out by densimetric separation and electronic sieve shaker, but this process is only carried out in export-type coffees due to the high cost. Or dilution in batches of better-quality coffees, which in turn will generate a product that has low market value, but meets current legislation (de Almeida and Spers, 2020; Kalschne et al., 2018; Santos and Nantes, 2014; Toci and Farah, 2014).

However, these low-quality coffees have the potential to be used for other purposes besides foods, such for example: as fertilizers, fuels, adsorbents, as a source of enzymes, among others (Durán et al., 2017; Esquivel and Jiménez, 2012; Jenkins et al., 2017; Rodrigues and Bragagnolo, 2013; Wei and Tanokura, 2015). They can also be used as a source of raw material to produce fixed oil, rich in polyphenolic compounds, kahweol, cafestol, tocopherols, in addition oleic, linoleic, and palmitic fatty acids (Acevedo et al., 2013; Esquivel Rodríguez et al., 2020; Jham et al., 2007; Oliveira et al., 2020; Ren et al., 2019; Tsukui et al., 2014).

Several applications for green coffee fixed oils in the pharmaceutical and cosmetic industries have been proposed. Wagemaker et al. (2012), Chiari et al. (2014), Nosari et al. (2015), and Acevedo et al. (2013) have demonstrated *in vitro* antioxidant activities of green coffee fixed oil and related it to preventing cellular skin damage. *In vitro* tests performed by Chiari et al. (2014) demonstrated that green coffee fixed oil has a synergistic effect when combined with a conventional synthetic sunscreen (ethyl-hexyl-methoxycinnamate) by increasing 20% the sun protection factor (SPF). *In vivo* tests performed on humans with formulations containing up to 15% (w/w) of green coffee fixed oils showed a significant reduction in transepidermal water loss (TEWL) after application for three days. The same formulations were also applied to the skin on the back of the volunteers using occlusive adhesive for two days, and no adverse reactions were observed (Wagemaker et al., 2013, 2015).

Studies carried out by Pereda et al. (2009) identified a dose-dependent relationship on the stimuli in the synthesis of collagen by green coffee oil; production of elastin and glycosaminoglycans by fibroblasts *in vitro*; greater stimuli to the growth factors (TGF-b1 and GM-CSF), and a positive association with the expression of aquaglyceroporins (AQP3) in keratinocytes, responsible for water transport and hydration in human skin epidermis. Low cytotoxicity of green coffee oil at models tested *in vitro* on human keratinocyte (HaCat) and human hepatoma (HepG2) cultures with a cell density of 1 x 10^6 cells/ml in formulations (10-100 µL green coffee fixed oils/ml, 24 h incubation, 3-4,5-dimethyl-thiazole-2-il-2,5-diphenyltrazzole bromide reduction assay) was reported (Wagemaker et al., 2013; Chiari et al., 2014).

Based on this information, the option of using low-quality coffees to obtain vegetable oil intends to use it as a cosmetic ingredient is visualized, which adds value to the coffee production chain by obtaining a by-product with greater market value and an abundant source of raw, especially in Brazil. In addition, it corroborates with the evidence that vegetable oils have attractive, functional properties for the pharmaceutical and cosmetic sectors, which have great interest in seeking new excipients and active ingredients from natural sources to replace compounds of synthetic origin (Ahmad and Ahsan, 2020; Bera et al., 2006; Blasi and Cossignani, 2020; Cruz et al., 2007; Garg et al., 2021; López-Barrera et al., 2016; Lourenço et al., 2019; Milatovic et al., 2016; Scott et al., 2020; Taghvaei and Jafari, 2015; Xu et al., 2015).

Thus, the objective of this work was to extract and characterize the fixed oil from non-beverage type green coffee beans and perform a preliminary evaluation of its functionality as a pharmaceutical ingredient.

2. Materials and methods

2.1. Raw materials

The fixed oil was obtained from green conilon coffee beans (GCC) classified as non-beverage type, with more than 50 black beans in 300 g of samples and impurity content of 12.6%. The sample was provided by the Cooperative of Coffee Growers of the South of Espírito Santo (CAFESUL), Brazil, and classified in accordance with the Official Brazilian Classification, following Normative Instruction No. 8/2003 of the Identity and Quality Technical Regulations for Processed Coffee Classification (Raw Grains) (Brasil, 2003; Brasil, 2020b). The other excipients of pharmaceutical-grade used to prepare the formulations were: Polawax®, decyl oleate, glyceryl monostearate, isopropyl myristate, and propylene glycol (Mix das Essências), liquid petrolatum (Farmax), methylparaben (Vetec), propylparaben (Êxodo), ethylenediaminetetraacetic acid disodium (Dinâmica) and butylated hydroxytoluene (BHT) (Vetec).

2.2. Obtaining the green conilon coffee fixed oil (CFO)

To obtain the fixed oil, the GCC was dried in an air circulation oven at 55 °C, crushed in a micro-Wiley knife mill (MARCONI, model MA 048), with a sieve of 20 mesh opening and subjected to extraction by Soxhlet (FISATOM, model 22/6, Brazil) with ethyl ether as solvent. The oil was centrifuged at 2.500 rpm (25 ± 2 °C, 5 min) in a centrifuge (HERMLE Labortechik GmbH, model Z206, Germany), filtered in qualitative filter paper, and refrigerated at -20 °C until analysis and preparation of the creams. The oil obtained was named CFO.

2.3. Composition and physicochemical properties of CFO

The physicochemical properties (refraction index-RI, acidity index, peroxide index, density, and the ultraviolet absorption coefficients at 232 nm and 270 nm) were investigated according to standard methods described in the guidelines of the Instituto Adolfo Lutz (2008).

Total tocopherol determination followed the methodology proposed by Durán et al. (2004) and Otemuyiwa and Adewusi (2013) with modifications. The oil samples were diluted in ethanol at a 1:10 ratio. A 1.0 ml aliquot was mixed with a 10^{-3} mol/l ferric chloride solution and stirred for 1 min. Then, 2.0 ml of acetic acid/sodium acetate buffer (pH = 3.3), 2.5 ml of 0.3% orthophenanthroline (m/v), and 0.2 ml of 10^{-3} mol/l phosphoric acid were added. The system was homogenized and kept in the dark for 10 min, followed by an absorbance reading at 534 nm in a UV-Vis spectrophotometer (EDUTEC, model EEQ-9023, Brazil). Quantification was performed by constructing a calibration curve prepared with an α-tocopherol standard (Merck, DL-α-Tocopherol, purity ≥ 98%) in ethanol.

Total phenolic compound content was determined by the Folin-Ciocalteu method according to Singleton et al. (1999). The absorbance was measured at 760 nm on a UV-Vis spectrophotometer (EDUTEC, model EEQ-9023). Quantification was performed by constructing a calibration curve with a gallic acid standard (Dinâmica, AG, purity ≥ 99%) in methanol.

The chromatographic profile was analyzed to determine the oils grease composition. The samples were derivatized and analyzed by gas chromatography coupled to mass spectrometry (GC-MS) in Agilent Technologies gas chromatography (GC 7890A, USA) equipped with a mass detector and DB-23 capillary column, 60 m length x 0.25 mm inner diameter x 0.25 µm film thickness. The identification of the components from fixed oils was performed by comparing the mass spectra of the device database (NIST 2.0) with data from the literature and also with the injection of standard substance solutions. The relative percentage of each compound was calculated by the ratio between the integral area of their respective peaks and the total area of all sample constituents.

2.4. In vitro antioxidant activity

The antioxidant activity of the CFO was determined by an extinction method for 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995; Ndayishimiye and Chun, 2017) with modifications (de Lima Souza et al., 2021). The absorbance was measured at 515 nm using a spectrophotometer (UV-VIS EDUTEC, model EEQ-9023), and the percentage of DPPH sequestration was calculated (Equation 1). The effective concentrations (IC_{50} and IC_{90}) of the oil and the BHT samples were estimated by a predefined tendency curve obtained in Microsoft Excel.

$$DPPH\ scavenging\ (\%) = 100 \times [(A_0-A_n)/(A_0)] \quad (1)$$

Where A_0 = control absorbance (no antioxidant) and A_n = sample absorbance at concentration n.

2.5. Pharmaceutical formulations of the creams

Three formulations using self-emulsifying non-ionic wax were prepared (F1, F2, and F3), with modifications described in the National Formulary from Brazilian Pharmacopeia (Brasil, 2012). A system of conventional synthetic emollients was used in the oil phase in F1: decyl oleate, isopropyl myristate, and liquid petrolatum. In F2, liquid petrolatum was replaced by CFO, and in F3, liquid petrolatum and decyl oleate was replaced by CFO. BHT was used as an antioxidant in F1 and was replaced by CFO in F2 and F3. The composition and denominations of the formulations are listed in Table 1.

Table 1. Formulations designed to evaluate the use of the CFO as pharmaceutical ingredients

Components	Formulation*		
	F1	F2	F3
% Oil phase (w/w)			
Self-emulsifying non-ionic wax (Polawax®)	11.00	11.00	11.00
Glyceryl monostearate	2.00	2.00	2.00
Isopropyl myristate	2.50	2.50	2.50
Decyl oleate	2.50	2.50	-
Liquid petrolatum	2.50	-	-
Butylated hydroxytoluene (BHT)	0.10	-	-
Green conilon coffee fixed oil (CFO)	-	2.50	5.00
% Aqueous phase (w/w)			
Propylene glycol	5.00	5.00	5.00
Methylparaben	0.15	0.15	0.15
Propylparaben	0.05	0.05	0.05
Ethylenediaminetetraacetic acid disodium	0.25	0.25	0.25
Purified water qs to	100	100	100

*Formulations described in the Brazilian Pharmacopeia National Formulary (Brasil, 2012), with adaptations.

The formulations were prepared on a laboratory scale, using a conventional emulsification process according to the National Formulary from Brazilian Pharmacopeia (Brasil, 2012), with modifications. The components of the aqueous and oily phase were heated separately to a temperature of 70-75 °C; the aqueous phase was added to the oily phase and subjected to moderate and continuous stirring in a domestic mixer (BRITANIA, model 200, Brazil) until complete cooling.

2.6. Analysis of the quality and preliminary physical stability of the creams

The description of the aspect and the formation of the emulsified systems were researched by macroscopic and microscopic analysis (Hu et al., 2017). The organoleptic characteristics of the creams (appearance, color, brightness, odor, texture, skin coverage, and residual color after application) were analyzed. The presence of phase separation was also observed. Microscopic evaluation was performed using an optical microscope (LEICA, model DM 500, Germany) with a built-in camera (LEICA, model ICC50 HD) at 40x magnification.

To assess the potential of using fixed oils as excipients, the prepared formulations were subjected to tests provided by ANVISA's cosmetic formulations quality and physical stability guidelines (Brasil, 2004). The pH was measured by direct potentiometry (MS-TECNOPON, model mPA-220, Brazil). To predict physical stability, the creams were subjected to different temperature conditions (40, 45, 50, 55, and 60 °C; during 15 min each cycle) and centrifugation cycles (1.000, 2.500, and 3.500 rpm; during 15 min each cycle).

Another important parameter that influences the acceptance of the formulations is the spreadability related to the sensorial properties of semi-solid formulations. The spreadability was determined by the parallel plate method according to the methodology described by Montenegro et al. (2015). Weighed 0.15 g of each formulation evenly in the center of a glass plate of known dimensions properly positioned on graph paper. Then, another glass plate of the same size and previously weighed was placed carefully above the plate containing the formulation. Then, known weights were added in sequence, and the values of the diameters reached by the sample corresponding to the weights were measured, and the area of the scattered sample was calculated (Equation 2).

The spreadability factor was calculated using the ratio between the maximum spreadability and the limit effort (Equation 3). To evaluate the spreadability behavior of the creams, graphs of spreadability versus strain (weight to which the sample was submitted) its first derivative was constructed.

$E_i = (\pi d^2)/4$ (2)

$Spreadability\ factor\ (mm^2/g) = (E_{max}/m_{max})$ (3)

Where E_i = spreadability of the sample for a given weight (mm^2); d = average diameter (mm), E_{max} = maximum spreadability of the sample, from which the product no longer spreads even when more effort is applied (mm^2) and m_{max} = corresponding weight to obtain maximum spreadability (g).

The method of compression of a sample between parallel plates was used to estimate the behavior of a fluid (Montenegro et al., 2015; Mezger, 2020). For this, the compression and its deformation were calculated from the Equations (4, 5), and the behavior of each cream was evaluated using compression versus volumetric deformation graphs.

$Compression\ (Pa) = (P_n/A)$ (4)

$Volumetric\ Strain = (\Delta V_n/V_0)$ (5)

Where P_n = corresponds to the accumulated weight with each new mass addition multiplied by the acceleration of gravity; A = glass plate area (m^2); ΔV_n = volume variation of cream corresponding to the masse addition; V_0 = initial volume of the cream.

2.7. Potentiality evaluation of CFO oil as an antioxidant in formulations

The potential use of CFO as an antioxidant in creams formulations instead of BHT was investigated by studying *in vitro* antioxidant activity, according to the previously described methodology for the fixed oil (Brand-Williams et al., 1995; Ndayishimiye and Chun, 2017). DPPH was diluted in absolute ethanol at a 1:20 ratio.

2.8. Statistical data analysis

The results obtained for oil extraction yield, physicochemical properties, chemical composition, and antioxidant activity of the oil and creams were subjected to descriptive analysis with a minimum of three experimental repetitions and expressed as mean ± standard deviation, calculated by the packages ExpDes, MASS, and STATS, program R, version 3.5.1 (R: Development Core Team, 2019). The chromatographic profile was carried out by a single repetition. The IC_{50} and IC_{90} values were estimated by the predefined tendency curve obtained in Microsoft Excel, using the best fit model.

3. Results and discussion

In the last few decades, an increase in interest in natural products can be noticed, as well as a growing consumer market worldwide for "green products", which are formulated with natural ingredients. The exclusion of traditional quality control methods based on animal testing and the encouragement to search for natural raw materials from renewable sources ensure the success of the "green cosmetics" market (Fonseca-Santos et al., 2015). The use of non-beverage type green coffee beans in the production of natural ingredients for use in the "green market", satisfies the interest of industries and consumers and adds value to the product regarding the coffee agribusiness. The oil obtained from the green conilon coffee presented, in its composition, high levels of fatty acids with emulsifying and emollient properties (palmitic acid, linoleic acid) and compounds with antioxidant functional properties (tocopherols and phenolic compounds). When incorporated into cream formulations, this oil has proven useful to be used as emollients and antioxidants in place of liquid petroleum jelly, decyl oleate, and BHT, widely used synthetic ingredients.

3.1. Physical classification of GCC

Physical classification is one of the tools responsible for assessing the quality of grains. The physical classification defines the type of coffee, ranked on a scale of two to seven or non-beverage type, the marketing of the latter being prohibited for not meeting the quality standards determined in Normative Instruction 08/2003 of Ministério da Agricultura, Pecuária e Abastecimento-MAPA (Brasil, 2003; Brasil, 2020b). The coffee used in this work presented more than 50 black grains in 300 g of sample, which classifies it as non-beverage type, and content of impurities above 1.0% that disqualifies it for commercialization unless it is processed again.

To be sold in the domestic or foreign market for food purposes, coffees with similar classifications to those analyzed in this work need to go through the re-processing of the beans, carried out by densimetric separation and electronic sieve shaker, at the cost of U$ 2.00 per bag of 60 kg (Cafesul, 2021), however, this process is only carried out in export-type coffees, due to the high cost. If not, then they must be diluted in batches of better-quality coffees, which it will result in a product with low market value, but that meets the current legislation (de Almeida and Spers, 2020; Kalschne et al., 2018; Santos and Nantes, 2014; Toci and Farah, 2014).

Based on this assessment, the search for more viable ways to use these low-quality coffees is suggested. Among the possibilities of use for the grains would be as a source of raw material for the extraction of fixed oil (Acevedo et al., 2013; Chiari et al., 2014; Nosari et al., 2015; Pereda et al., 2009; Tsukui et al., 2014; Wagemaker et al., 2012, 2013, 2015; Xu et al., 2015).

3.2. Obtention and physicochemical characterization of CFO

After drying in an air circulation oven at 55 °C, the GCC sample showed 3.92 ± 0.12% (w/w) moisture content. Extraction of GCC by the Soxhlet device with ethyl ether as solvent produced a yield of 3.70 ± 1.29% (w/w) in fixed oil (CFO). This yield can be considered low when compared to those obtained by other authors for good quality varieties of *C. canephora* (2-12% w/w) (Aguiar et al., 2005; Brige, 2016; Mazzafera et al., 1998; Kemsley et al., 1995), which was already expected because the sample had a high content of impurities (12.60%). However, considering the market value (U$ 100.00/kilogram of fixed coffee oil), the quantity obtained was expressive.

The CFO obtained from GCC was liquid at room temperature, clear to slightly cloudy, brownish-green to amber in color, with a characteristic odor of green coffee beans, density at 20 °C (0.9117 ± 0.0027 g/cm^3), and refractive index at 40 °C (1.4630 ± 0.0015 g/cm^3). Despite the large number of impurities contained in GCC, the density (D) and refractive index (RI) values of CFO did not differ much from those found by other authors in quality coffees (D = 0.9157-0.9418 and IR = 1.4300-1.4810) (Abdullah and Koc, 2013; Al-Hamamre et al., 2012; de Oliveira et al., 2014; Oliveira et al., 2020; Somnuk et al., 2017).

The acidity and peroxide index parameters make it possible to assess the conservation status of fixed vegetable oils since lipid decomposition is almost always accompanied by the formation of free fatty acids and peroxides (Hosseini et al., 2016; Instituto Adolfo Lutz, 2008). According to Anvisa (Brasil, 2005) and Codex Standards for fats and oils from vegetable sources (FAO, 2021), crude or

unrefined oils must have a maximum of 4.0 mg KOH/g acidity index and 15 meq/kg peroxide index. The CFO showed an acidity index (3.61 ± 0.08 mg KOH/g) and a peroxide index (138.31 ± 15.31 meq/kg). These results indicate high levels of lipid decomposition products.

The presence of lipid decomposition products in the CFO was also verified by measuring the extinction coefficient at 232 nm (4.46 ± 0.07) and at 270 nm (19.68 ± 0.08). A high absorption in 232 nm region indicates the presence of conjugated dienes, which are produced from the degradation of polyunsaturated fatty acids, and high absorption in 270 nm indicates the presence of aldehydes and ketones, a result of lipid oxidation (Dantas et al. 2011; Ferrari and Souza, 2009).

3.3. Chemical characterization of CFO

The CFO showed in its saponifiable fraction predominance of palmitic and linoleic acids. The unsaponifiable fraction showed considerable levels of tocopherols and phenolic compounds (Table 2).

Table 2. Chemical composition of green conilon coffee fixed oil (CFO)

Fatty acids	RT (min)	Relative area (%)*	Literature values***
Palmitic (C16:0)	13.346	47.76	27.2-32.1
Linoleic (C18:2 $\Delta^{9,12}$)	16.357	32.98	43.9-49.3
Oleic (C18:1 Δ^{9})	16.509	7.66	9.7-14.2
Stearic (C18:0)	17.009	9.55	5.8-8.0
Arachidic (C20:0)	21.097	2.05	2.1-4.4
Insaponification compounds		**Content (mg/kg)****	
Total tocopherols		788.71 ± 56.08	
Total phenolics		3312.40 ± 14.62	

*Values were determined by using gas chromatography with mass detector (CG-EM) and capillary column DB-23, with single injection of the samples and patterns.
**Values are means ± SD of three determinations (*n*=3). Abbreviations: CFO, green conilon coffee fixed oil obtained by solvent extraction with ethyl ether.
***(D'Amelio et al., 2013; Górnaś et al., 2014; Górnaś et al., 2016; Speer and Kölling-Speer, 2006; Wagemaker et al., 2011)

The presence of saturated and unsaturated fatty acids in the constitution of fixed oils attributes emollient properties when they are incorporated into cosmetic formulations (Pereira et al., 2005). Emollient oils containing these acids are incorporated on the keratinocytes of the epidermis, favoring the maintenance and regeneration of the lipidic barrier and, contributing to the formation of an occlusive layer, preventing the transepidermal water loss (TEWL) (do Rosário et al., 2021; Lu et al., 2011; Sarkar et al., 2017; Zielińska and Nowak, 2017). Additionally, the fatty-acid components of vegetable oils, polyunsaturated fatty acids such as linoleic and linolenic, have a pro-inflammatory effect and can accelerate the healing process (Poljšak et al., 2020). Palmitic and stearic acids are also used by the cosmetic industry to manufacture formulations due to their emulsifying and stabilizing properties (Rosiaux et al., 2015; Sarkar et al., 2017; Zielińska and Nowak, 2017).

In the unsaponifiable fraction of the CFO, the presence of the aforementioned bioactive compounds was identified. Tocopherols, precursors of vitamin E, considered a protective vitamin for the skin due to their powerful antioxidant capacity, act as scavengers of free radicals, protecting the skin from the harmful effects of solar radiation and haves emollient properties, promoting skin hydration (Keen and Hassan, 2016). Phenolic compounds help to improve the immune system, preventing degenerative progressions such as cancer, cataracts, neurological diseases, cardiovascular diseases, and chronic diseases (do Rosário et al., 2021; Lu et al., 2011; Rosiaux et al., 2015; Sarkar et al., 2017; Zielińska and Nowak, 2017).

3.4. Antioxidant activity of CFO

The CFO showed antioxidant activity against the free radical DPPH in all tested concentrations, reaching 50% inhibition (IC_{50}) in the concentration of 0.59 mg/ml and 90% (IC_{90}) in 0.96 mg/ml. The sequestering behavior of the CFO was linear, and at concentrations ≥ 1.0 mg/ml, the antioxidant action was similar to BHT 0.20 mg/ml (Figure 1).

The IC_{50} and IC_{90} values of the CFO were higher than those of the BHT (0.04 and 0.2 mg/ml), which requires a greater amount of CFO to inhibit the free radical DPPH. However, it must be considered that the active antioxidant compounds present in the CFO, tocopherols, and phenolic compounds (Table 2) are from a natural source and offer low risk to human health.

3.5. Preliminary stability studies in creams formulations prepared with CFO

Creams are semi-solid dosage forms obtained from the emulsification of two phases (aqueous and oily) in the presence of one or more emulsifiers, under heating and with stirring (Allen Jr et al., 2013). Therefore, it has peculiar characteristics. Preliminary stability studies can be carried out during the formulation phase of a product to guide the choice between different formulations and evaluate the need for modifications to adapt them (Brasil, 2004). The tests performed to determine the preliminary stability in the present work were the aspect description, pH determination, thermal and mechanical stress, and spreadability.

When analyzed with the naked eye, all proposed formulations were smooth and lump-free with no evidence of creaming, flocculation, or coalescence. The F1 formulation showed milk-white color, and formulations prepared with CFO (F2 and F3) showed a beige-white color. When analyzed macroscopically, no creaming, flocculation, or coalescence was noted. The odor was characteristic of the raw materials used, with no rancid odor or of the solvent used to extract the oil.

Regarding the sensory aspect on the skin, the formulation prepared with the conventional emollient system (F1) presented less dense, with a tackiness touch, high gloss, low coverage, thin layer, and white on the skin after application disappeared within a few seconds. The formulation in which liquid petrolatum, decyl oleate, and BHT were replaced by CFO (F3) presented denser, with a dry touch, the lower gloss, high coverage, and transparency on the skin after application. The formulation in which liquid petrolatum and BHT were replaced by CFO (F2) presented intermediate characteristics.

In the photograph analysis of optical microscopy, individual spherical droplets dispersed in the medium were observed (Figure

2). A reduction in droplet size was observed in formulation with a higher proportion of fixed oil (F3). Such observations suggest that this formulation has a greater tendency to physical stability since the drop size correlates with the physical stability of the emulsion. When emulsified systems do not tend to flocculate, the observed drops are uniformly distributed and small; in emulsions that tend to flocculate, the droplets are larger and are close to each other, without merging into bigger ones (Allen Jr et al., 2013; Hu et al., 2017).

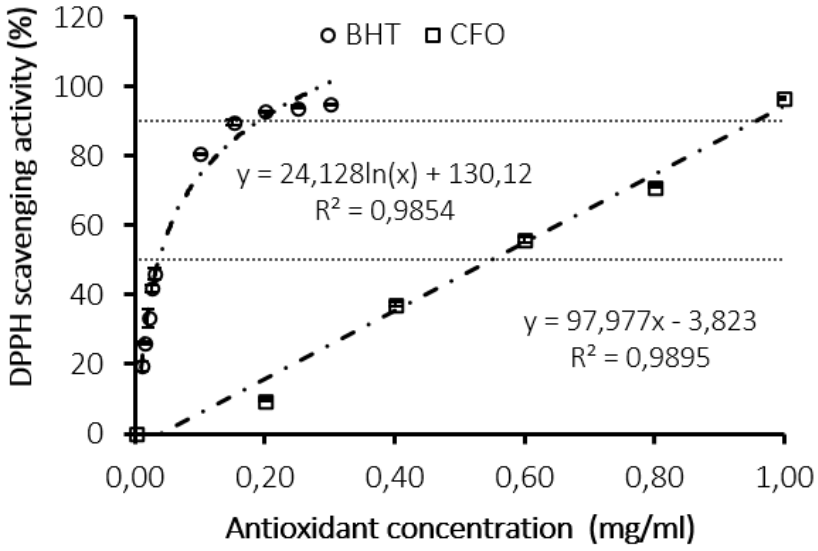

Figure 1. Antioxidant activity of BHT and CFO
Values are means ± SD of three determinations (*n*=3).
Abbreviations: BHT-butylated hydroxytoluene, and CFO-green conilon coffee fixed oil obtained by solvent extraction with ethyl ether

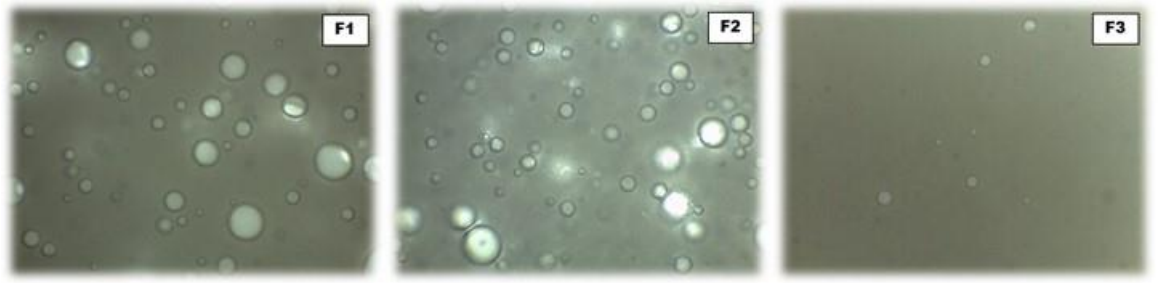

Figure 2. Microphotographs of creams prepared in a single batch each, under 40× magnification
F1: Cream with isopropyl myristate emollient system, liquid petrolatum and decyl oleate
F2: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent to replacing liquid petrolatum
F3: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent, to replace liquid petrolatum, decyl oleate and BHT

A factor of great relevance for the acceptance of cosmetic products by consumers is the sensory aspect, among which is the ease of spreading and removal (Allen Jr et al., 2013; Aulton and Taylor, 2016). The F1 and F2 formulations presented the highest spreadability factors (23.2 ± 0.4 and 26.2 ± 0.5 mm^2/g, respectively), being the easiest to spread. In F3, the lowest value was observed (5.5 ± 0.6 mm^2/g), suggesting it was more difficult to spread. F1 and F2 formulations showed more pronounced variations in spreadability at the beginning of the application of the masses (*f*'1 and *f*'2) and tending to constancy, and F3 formulation practically did not vary the spreadability with the increase in the application of the masses (*f*'3) (Figure 3A).

The replacement of liquid petrolatum by CFO in F2 effectively increased the spreadability of the formulation, while the replacement of decyl oleate and liquid petrolatum by CFO in F3 provided the formulation with a decrease. The first one is indicated for cosmetic products submitted to small compressions, with minimum effort conditions required for application to the skin. The last one is indicated for cosmetic products that are used to cover fine lines, facial sunscreen, and makeup in general, as they can be subject to great tensions, with the high effort required for application to the skin, but providing a more uniform coverage (Aulton and Taylor, 2016; Parente et al., 2005).

When evaluated in relation to the volumetric compression-deformation effect, a similar behavior was observed in formulations F1 and F2; both behaved as non-Newtonian pseudoplastic fluids, becoming more fluid when subjected to high stresses, while F3 showed a characteristic behavior of Newtonian fluid with a proportional increase between the volumetric strain and the compression rate (Figure 3B). This characteristic may be associated with the presence of air droplets in the F1 and F2 creams (Figure 2), which are removed with compression, causing an increase in fluidity, due to structural changes resulting from the applied forces (Attwood et al., 2011; Dak et al., 2007; Shamsudin et al., 2013). Meanwhile, in F3, these changes are less pronounced due to smaller droplet size and greater emulsion homogeneity (Figure 3) with constant cream fluidity, regardless of the applied force (Allen Jr et al., 2013).

Another important quality parameter for topical products is pH determination. The pH values of the creams were in accordance with the normal cutaneous pH, which corresponds to a range of 4 to 6.5 (Proksch, 2018). The incorporation of CFO in the formulation in a 2.5% w/w proportion did not affect the pH of the formulation since F1 and F2 presented similar pH values. In the proportion of 5.0%, there was a significant reduction in the pH of the formulation, observed in F3. This decrease in the pH values of formulations where there was a total replacement of liquid petroleum jelly and decyl oleate emollients is mainly associated with the increase of the CFO content in the formulation and, consequently, of free fatty acids, since part of the carboxylic groups present in fatty acids tend to ionize at the interface of the emulsion droplets (Bruxel et al., 2012; Rabinovich-Guilatt et al., 2005).

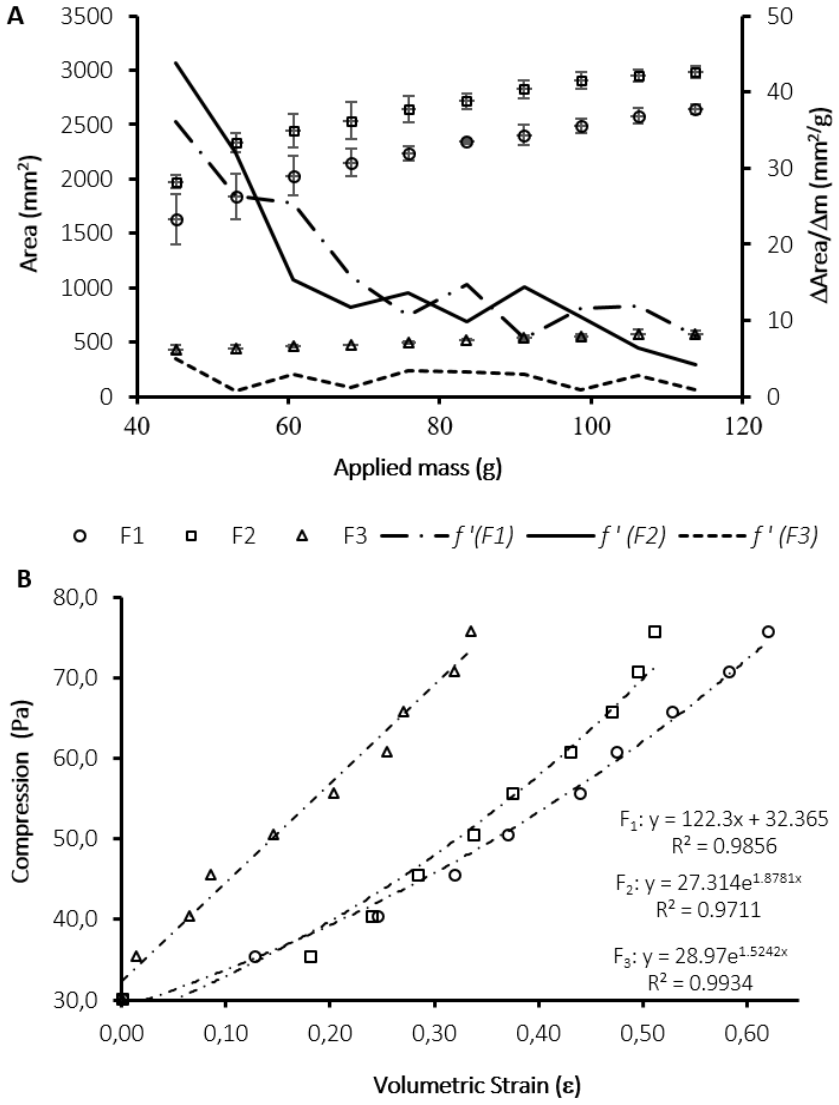

Figure 3. Creams spread profile using the parallel plate method
F1: Cream with isopropyl myristate emollient system, liquid petrolatum and decyl oleate
F2: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent to replacing liquid petrolatum
F3: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent, to replace liquid petrolatum, decyl oleate and BHT

Finally, formulations were submitted to thermal and mechanical stress. None of the formulations showed signs of flocculation, sedimentation, and creaming formation. However, formulations F1 and F2 showed an increase in fluidity from 50 °C, and F2 presented air bubbles at the bottom of the tube when subjected to centrifugation, starting at 1.000 rpm. These changes are probably due to the sedimentation phenomenon because the test is based on separating phases with different densities (Mohsin et al., 2016). Formulation F3 did not show any type of alteration, which may indicate greater physical stability, which is in accordance with the images observed by microscopy (Figure 2).

3.6. Antioxidant activity of CFO in creams

The possibility of replacing BHT by CFO in formulations becomes quite attractive due to the fact of replacing a synthetic component with a natural one, and also due to the concentration limits of BHT recommended by international regulatory authorities for their presence in topical use formulations (0.0075-0.1% w/w) (Rowe et al., 2009).

Figure 4A expresses the CFO antioxidant activity present in the creams in terms of DPPH sequestration: F1 (16.2 ± 0.3%), F2 (44.5 ± 1.1%), and F3 (44.6 ± 0.8%). Greater efficiency was observed in

scavenging the free radical DPPH in formulations containing CFO as an antioxidant (F2 and F3) than in the formulation with BHT (F1).

The effective concentrations for 50% inhibition of the free radicals DPPH were: F1 (0.07 mg/ml of BHT), F2 (0.13 mg/ml of CFO) and F3 (0.26 mg/ml of CFO) (Figure 4B). These results indicate that incorporating CFO in both cream formulations at the concentrations tested is sufficient to replace the use of BHT as an antioxidant. This converges with evidence that natural antioxidants can present antioxidant activity comparable to synthetic antioxidants (Ahmad and Ahsan, 2020; Bera et al., 2006; Blasi and Cossignani, 2020, Cruz et al., 2007; Garg et al., 2021; López-Barrera et al., 2016; Lourenço et al., 2019; Milatovic et al., 2016; Taghvaei and Jafari, 2015; Xu et al., 2015).

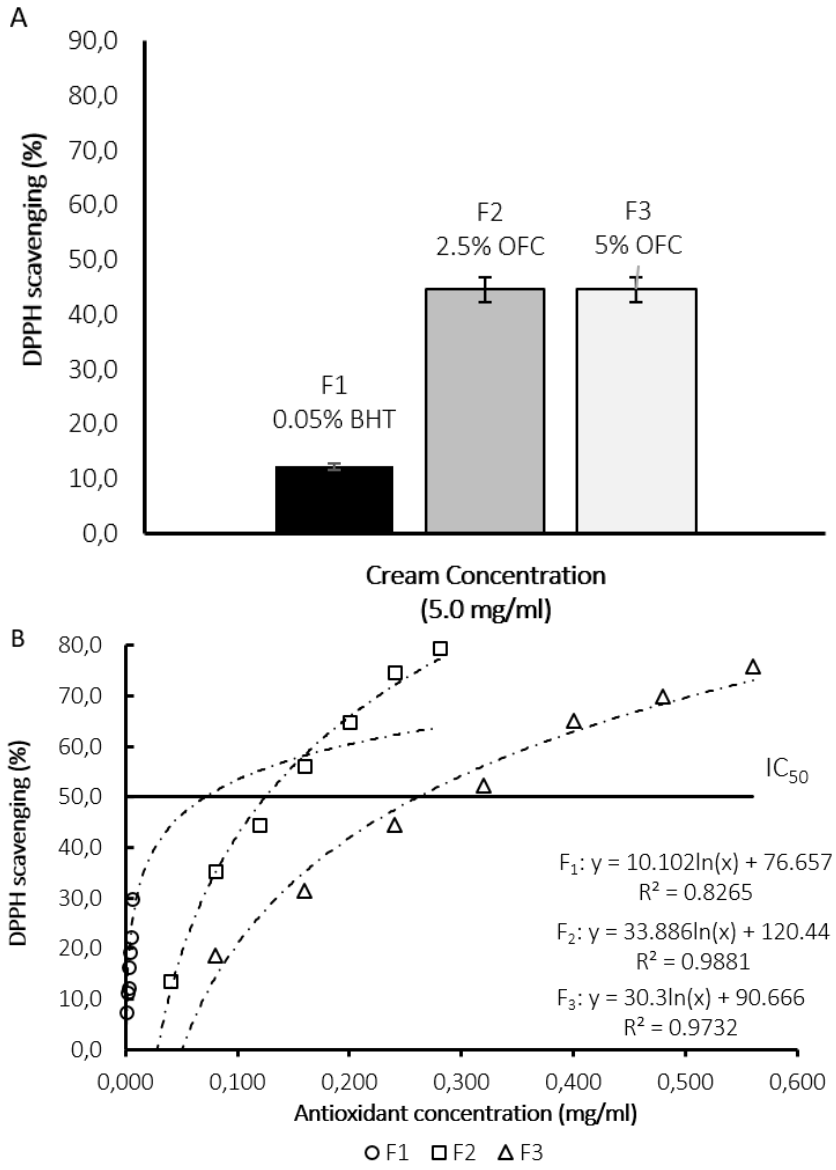

Figure 4. DPPH scavenging by the action of creams, and comparative behavior related of the antioxidant concentration present in each formulation

F1: Cream with isopropyl myristate emollient system, liquid petrolatum and decyl oleate
F2: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent to replacing liquid petrolatum
F3: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent, to replace liquid petrolatum, decyl oleate and BHT
Effective concentrations-IC_{50}

For active antioxidant purposes, the increase in CFO concentration, F2-2.5% (w/w) and F3-5.0% (w/w), provided no difference in DPPH sequestration activity, showing similar behavior (Figure 5). This result shows that the presence of the CFO compounds with antioxidant action in the formulation (F2 and F3) is not only related to their concentration but depends on a variety of physicochemical factors, including pH, the viscosity of the continuous phase, droplet size distribution, oil/water ratio and temperature (Frelichowska et al., 2009; Salmela and Washington, 2014; Spernath et al., 2008).

4. Conclusions

Increasingly, consumers are looking for products or products based on ingredients that have less impact on the environment, including cosmetics. Formulating "eco-friendly" cosmetics is a challenge for formulators who must be able to guarantee stability, safety, and efficiency to the product. Preliminary CFO analysis obtained from non-beverage type green coffee beans showed that the fixed oil was promising in preparing creamy foundations, replacing synthetic

emollients, liquid petroleum jelly, and decyl oleate, and replacing the BHT synthetic antioxidant. Creams with 5% w/w of CFO (F3) showed aspect, droplets size, pH, spreadability, and thermal and mechanical stability profile according to guidelines recommendations.

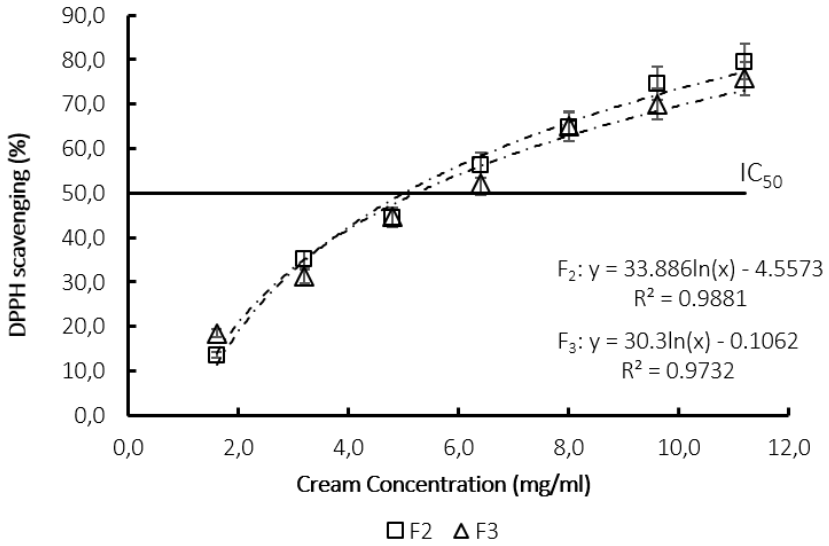

Figure 5. Comparison of DPPH scavenging of the creams
F1: Cream with isopropyl myristate emollient system, liquid petrolatum and decyl oleate
F2: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent to replacing liquid petrolatum
F3: Cream incorporated with green conilon coffee fixed oil obtained by extraction with ethyl ether solvent, to replace liquid petrolatum, decyl oleate and BHT
Effective concentrations-IC_{50}

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Conflict of interest

The authors declare that they have no known competing financial interests or relationships that could have influenced the work reported in this paper.

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Supplementary File

None.

References

Abdullah, M., Koc, A.B., 2013. Oil removal from waste coffee grounds using two-phase solvent extraction enhanced with ultrasonication. *Renewable Energy*, 50, 965-970.
Acevedo, F., Rubilar, M., Scheuermann, E., Cancino, B., Uquiche, E., Garcés, M., Shene, C., 2013. Spent coffee grounds as a renewable source of bioactive compounds. *Journal of Biobased Materials and Bioenergy*, 7(3), 420-428.
Aguiar, A.T.D.E., Salva, T.D.J.G., Fazuoli, L.C., Favarin, J.L., 2005. Variação no teor de lipídios em grãos de variedades de *Coffea canephora*. *Pesquisa Agropecuária Brasileira*, 40, 1251-1254.
Ahmad, A., Ahsan, H., 2020. Lipid-based formulations in cosmeceuticals and biopharmaceuticals. *Biomedical Dermatology*, 4(1), 1-10.
Al-Hamamre, Z., Foerster, S., Hartmann, F., Kröger, M., Kaltschmitt, M., 2012. Oil extracted from spent coffee grounds as a renewable source for fatty acid methyl ester manufacturing. *Fuel*, 96, 70-76.
Allen Jr, L.V., Popovich, N.G., Ansel, H.C., 2013. Formas Farmacêuticas e Sistemas de Liberação de Fármacos, nine ed. Artmed Editora, Porto Alegre. ISBN 9788565852845.
Attwood, D., Florence, A.T., Rothschild, Z., 2011. Princípios Físico-Químicos em Farmácia. Second ed. Farmabooks, São Paulo. ISBN 978858973135-5.
Aulton, M.E, Taylor, K.M.G., 2016. Delineamento de Formas Farmacêuticas, fourth ed, Elsevier, Brasil. ISBN.8535262784, 9788535262780.
Bera, D., Lahiri, D., Nag, A., 2006. Studies on a natural antioxidant for stabilization of edible oil and comparison with synthetic antioxidants. *Journal of Food Engineering*, 74(4), 542-545.
Blasi, F., Cossignani, L., 2020. An overview of natural extracts with antioxidant activity for the improvement of the oxidative stability and shelf life of edible oils. *Processes*, 8(8), 956.
Brand-Williams, W., Cuvelier, M.E., Berset, C.L.W.T., 1995. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, 28(1), 25-30.
Brasil, Anvisa, 2004. Agência Nacional de Vigilância Sanitária. Guia de estabilidade de produtos cosméticos. Editora Anvisa, 2004. ISBN 85-88233-15-0.
Brasil, Anvisa, 2005. Resolução RDC ANVISA/MS n°.270, de 22 de setembro de 2005. Regulamento Técnico para Óleos Vegetais, Gorduras Vegetais e Creme Vegetal.
Brasil, Anvisa., 2012. Formulário Nacional da Farmacopeia Brasileira. Edn 2, Agência Nacional de Vigilância Sanitária, Brasília. p. 224.

Brasil, Companhia Nacional de Abastecimento, 2020a. Acompanhamento da safra brasileira de café, v. 6 – Safra 2020, n. 4 - Quarto levantamento, Brasília, p. 1-45, dezembro 2020. ISSN 2318-7913.

Brasil, Ministério da Agricultura, Pecuária e Abastecimento, 2003. Instrução Normativa nº 8, de 11 de junho de 2003. Regulamento Técnico de Identidade e de Qualidade para a Classificação do Café Beneficiado Grão Cru. Diário Oficial da República Federativa do Brasil, Brasília, DF, 13 jun. 2003. Seção 1, p. 22-29.

Brasil, Ministério da Agricultura, Pecuária e Abastecimento, 2020b. Instrução Normativa nº 8, de 11 de junho de 2003. Regulamento Técnico de Identidade e de Qualidade para a Classificação do Café Beneficiado Grão Cru. Diário Oficial da República Federativa do Brasil. Brasília, DF, 15 set. 2020. Seção 1, p. 22-29.

Brige, F.A.A., 2016. Variabilidade genética e caracterização química de acessos de café conilon em sistema de cultivo irrigado no Cerrado. Disserrtaçao de Mestrado em Agronomia, Universidade de Brasilia.

Bruxel, F., Laux, M., Wild, L.B., Fraga, M., Koester, L.S., Teixeira, H.F., 2012. Nanoemulsões como sistemas de liberação parenteral de fármacos. *Química Nova*, 35(9), 1827-1840.

Cafesul, 2021. Cooperativa dos Cafeicultores do Sul do Estado do Espírito Santo.

Chiari, B.G., Trovatti, E., Pecoraro, É., Corrêa, M.A., Cicarelli, R.M.B., Ribeiro, S.J.L., Isaac, V.L.B., 2014. Synergistic effect of green coffee oil and synthetic sunscreen for health care application. *Industrial Crops and Products*, 52, 389-393.

Cruz, J.M., Conde, E., Domínguez, H., Parajó, J.C., 2007. Thermal stability of antioxidants obtained from wood and industrial wastes. *Food Chemistry*, 100(3), 1059-1064.

Dak, M., Verma, R.C., Jaaffrey, S.N.A., 2007. Effect of temperature and concentration on rheological properties of "Kesar" mango juice. *Journal of Food Engineering*, 80(4), 1011-1015.

D'Amelio, N., De Angelis, E., Navarini, L., Schievano, E., Mammi, S., 2013. Green coffee oil analysis by high-resolution nuclear magnetic resonance spectroscopy. *Talanta*, 110, 118-127.

Dantas, M.B., Albuquerque, A.R., Barros, A.K., Rodrigues Filho, M.G., Antoniosi Filho, N.R., Sinfrônio, F.S.M., Souza, A.G., 2011. Evaluation of the oxidative stability of corn biodiesel. *Fuel*, 90(2), 773-778.

de Almeida, L.F., Spers, E.E., 2020. Coffee Consumption and Industry Strategies in Brazil. Woodhead Publishing. ISBN: 978-0-12-814721-4.

de Lima Souza, J.R.C., Villanova, J.C.O., de Souza, T.D.S., Maximino, R.C., Menini, L., 2021. Vegetable fixed oils obtained from soursop agro-industrial waste: Extraction, characterization and preliminary evaluation of the functionality as pharmaceutical ingredients. *Environmental Technology & Innovation*, 21, 101379.

de Oliveira, P.M.A., de Almeida, R.H., de Oliveira, N.A., Bostyn, S., Goncalves, C.B., de Oliveira, A.L., 2014. Enrichment of diterpenes in green coffee oil using supercritical fluid extraction–Characterization and comparison with green coffee oil from pressing. *The Journal of Supercritical Fluids*, 95, 137-145.

do Rosário, M.S., Gauto, M.I.R., Silva, A.C.L.N., Sales, J.S., dos Santos Pereira, F., dos Santos, E.P., Costa, M.C.P., 2021. Estudo de estabilidade de emulsão cosmética com potencial de creme hidratante para o tratamento da xerose cutânea utilizando o óleo de babaçu (*Orbignya phalerata* Martius). *Brazilian Journal of Development*, 7(3), 29552-29570.

Durán, C.A., Regalado, E., Guzmán, M., Navas, P.B., 2004. Determinación de vitamina E en aceites. *Una experiencia didáctica en Química Analítica Instrumental. Educación Química*, 15(4e), 343-348.

Durán, C.A., Tsukui, A., Santos, F.K.F., Martinez, S.T., Bizzo, H.R., Rezende, C.M., 2017. Café: Aspectos Gerais e seu Aproveitamento para além da Bebida. *Revista Virtual de Química*, 9(1), 107-134.

Esquivel Rodríguez, P., Viñas Meneses, M., Steingass, C.B., Gruschwitz, M., Guevara Berger, E., Carle, R., Schweiggert, R.M., Jiménez García, V., 2020. V. Coffee (*Coffea arabica* L.) by-Products as a Source of Carotenoids and Phenolic Compounds—Evaluation of Varieties with Different Peel Color.

Esquivel, P., Jimenez, V.M., 2012. Functional properties of coffee and coffee by-products. *Food Research International*, 46(2), 488-495.

FAO (Food and Agriculture Organization of the United Nations), 2021. Codex Standards for Fats and Oils from Vegetable Sources. Accessed August 2021.

Ferrari, R.A., Souza, W.L.D., 2009. Avaliação da estabilidade oxidativa de biodiesel de óleo de girassol com antioxidantes. *Química Nova*, 32(1), 106-111.

Fonseca-Santos, B., Corrêa, M.A., Chorilli, M., 2015. Sustainability, natural and organic cosmetics: consumer, products, efficacy, toxicological and regulatory considerations. *Brazilian Journal of Pharmaceutical Sciences*, 51, 17-26.

Frelichowska, J., Bolzinger, M.A., Valour, J.P., Mouaziz, H., Pelletier, J., Chevalier, Y., 2009. Pickering w/o emulsions: drug release and topical delivery. *International Journal of Pharmaceutics*, 368(1-2), 7-15.

Garg, S.K., Shukla, A., Choudhury, S., 2021. Green coffee beans. In Nutraceuticals Academic Press, Cambridge, pp. 725-748.

Górnaś, P., Dwiecki, K., Siger, A., Tomaszewska-Gras, J., Michalak, M., Polewski, K., 2016. Contribution of phenolic acids isolated from green and roasted boiled-type coffee brews to total coffee antioxidant capacity. *European Food Research and Technology*, 242(5), 641-653.

Górnaś, P., Siger, A., Pugajeva, I., Czubinski, J., Waśkiewicz, A., Polewski, K., 2014. New insights regarding tocopherols in Arabica and Robusta species coffee beans: RP-UPLC-ESI/MSn and NP-HPLC/FLD study. *Journal of Food Composition and Analysis*, 36(1-2), 117-123.

Hameed A., Hussain S.A., Suleria H.A.R., 2018. "Coffee Bean-Related" Agroecological Factors Affecting the Coffee. In: Merillon JM., Ramawat K. (eds) Co-Evolution of Secondary Metabolites. Reference Series in Phytochemistry. Springer, Cham.

Hosseini, H., Ghorbani, M., Meshginfar, N., Mahoonak, A.S., 2016. A review on frying: procedure, fat, deterioration progress and health hazards. *Journal of the American Oil Chemists' Society*, 93(4), 445-466.

Hu, Y.T., Ting, Y., Hu, J.Y., Hsieh, S.C., 2017. Techniques and methods to study functional characteristics of emulsion systems. *Journal of Food and Drug Analysis*, 25(1), 16-26.

Instituto Adolfo Lutz (São Paulo), 2008. Métodos físico-químicos para análise de alimentos: /coordenadores Odair Zenebon, Neus Sadocco Pascuet e Paulo Tiglea -- São Paulo: Instituto Adolfo Lutz. versão eletrônica, 1020p.

Jenkins, R.W., Ellis, E.H., Lewis, E.J., Paterson, M., Le, C.D., Ting, V.P., Chuck, C.J., 2017. Production of biodiesel from Vietnamese waste coffee beans: biofuel yield, saturation and stability are all elevated compared with conventional coffee biodiesel. *Waste and Biomass Valorization*, 8(4), 1237-1245.

Jham, G.N., Winkler, J.K., Berhow, M.A., Vaughn, S.F., 2007. γ-Tocopherol as a marker of Brazilian coffee (*Coffea arabica* L.) adulteration by corn. *Journal of Agricultural and Food Chemistry*, 55(15), 5995-5999.

Kalschne, D.L., Viegas, M.C., De Conti, A.J., Corso, M.P., de Toledo Benassi, M., 2018. Steam pressure treatment of defective *Coffea canephora* beans improves the volatile profile and sensory acceptance of roasted coffee blends. *Food Research International*, 105, 393-402.

Keen, M.A., Hassan, I., 2016. Vitamin E in dermatology. *Indian Dermatology Online Journal*, 7(4), 311.

Kemsley, E.K., Ruault, S., Wilson, R.H., 1995. Discrimination between *Coffea arabica* and *Coffea canephora* variant robusta beans using infrared spectroscopy. *Food Chemistry*, 54(3), 321-326.

López-Barrera, D.M., Vázquez-Sánchez, K., Loarca-Piña, M.G.F., Campos-Vega, R., 2016. Spent coffee grounds, an innovative source of colonic fermentable compounds, inhibit inflammatory mediators *in vitro*. *Food Chemistry*, 212, 282-290.

Lourenço, S.C., Moldão-Martins, M., Alves, V.D., 2019. Antioxidants of natural plant origins: From sources to food industry applications. *Molecules*, 24(22), 4132.

Lu, C., Napier, J.A., Clemente, T.E., Cahoon, E.B., 2011. New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Current Opinion in Biotechnology*, 22(2), 252-259.

Mazzafera, P., Soave, D., Zullo, M.A.T., Guerreiro Filho, O., 1998. Oil content of green beans from some coffee species. *Bragantia*, 57(1), 45-48.

Mezger, T.G., 2020. The rheology handbook: for users of rotational and oscillatory rheometers. Fifth edition, European Coatings, Hanover. ISBN 978-3-86630-532-8.

Milatovic, D., Zaja-Milatovic, S., Gupta, R.C., 2016. Oxidative stress and excitotoxicity: antioxidants from nutraceuticals. In Nutraceuticals. Academic Press, Cambridge. 401-413.

Mohsin, S., Akhtar, N., Mahmood, T., Khan, H., Mustafa, R., 2016. Formulation and stability of topical water in oil emulsion containing corn silk extract. *Tropical Journal of Pharmaceutical Research*, 15(6), 1115-1121.

Montenegro, L., Rapisarda, L., Ministeri, C., Puglisi, G., 2015. Effects of lipids and emulsifiers on the physicochemical and sensory properties of cosmetic emulsions containing vitamin E. *Cosmetics*, 2(1), 35-47.

Ndayishimiye, J., Chun, B.S., 2017. Optimization of carotenoids and antioxidant activity of oils obtained from a co-extraction of citrus (*Yuzu ichandrin*) by-products using supercritical carbon dioxide. *Biomass and Bioenergy*, 106, 1-7.

Nosari, A.B., Lima, J.F., Serra, O.A., Freitas, L.A.P., 2015. Improved green coffee oil antioxidant activity for cosmetical purpose by spray drying microencapsulation. *Revista Brasileira de Farmacognosia*, 25, 307-311.

Oliveira, É.R., Carvalho, G.R., Cirillo, M.Â., Queiroz, F., 2020. Effect of ecofriendly bio-based solvents on oil extraction from green coffee bean and its industrial press cake. *Brazilian Journal of Chemical Engineering*, 36(4), 1739-1753.

Otemuyiwa, I.O., Adewusi, S.R., 2013. Fatty acid, carotenoid and tocopherol content of some fast foods from a Nigerian eatery. *Journal of Food and Nutrition Research*, 1(5), 82-86.

Parente, M.E., Ga´ Mbaro, A., Solana, G., 2005. Study of sensory properties of emollients used in cosmetics and their correlation with physicochemical properties. *International Journal of Cosmetic Science*, 27(6), 354-354.

Pereda, M.D.V., Dieamant, G., Eberlin, S., Nogueira, C., Colombi, Stasi, L.C.D., Queiroz, M.L.S., 2009. Effect of green *Coffea arabica* L. seed oil on extracellular matrix components and water-channel expression in *in vitro* and *ex vivo* human skin models. *Journal of Cosmetic Dermatology*, 8(1), 56-62.

Pereira, N.P., Miguel, O.G., Miguel, M.D., 2005. Chemical composition of the fixed oil from [*Chamomilla recutita* (L.) Rauschert] dried fruits native to the municipality of Mandirituba, Paraná (PR). *Revista Brasileira de Farmacognosia*, 15, 334-337.

Poljšak, N., Kreft, S., Kočevar Glavač, N., 2020. Vegetable butters and oils in skin wound healing: Scientific evidence for new opportunities in dermatology. *Phytotherapy Research*, 34(2), 254-269.

Poltronieri, P., Rossi, F., 2016. Challenges in specialty coffee processing and quality assurance. *Challenges*, 7(2), 19.

Proksch, E., 2018. pH in nature, humans and skin. *The Journal of Dermatology*, 45(9), 1044-1052.

R: Development Core Team, 2019. A Language and Environment for Statistical Computing. Version 3.6.2 (2019-12-12). The R Foundation for Statistical Computing.

Rabinovich-Guilatt, L., Dubernet, C., Gaudin, K., Lambert, G., Couvreur, P., Chaminade, P., 2005. Phospholipid hydrolysis in a pharmaceutical emulsion assessed by physicochemical parameters and a new analytical method. *European Journal of Pharmaceutics and Biopharmaceutics*, 61(1-2), 69-76.

Ren, Y., Wang, C., Xu, J., Wang, S., 2019. Cafestol and kahweol: A review on their bioactivities and pharmacological properties. *International Journal of Molecular Sciences*, 20(17), 4238.

Rodrigues, N.P., Bragagnolo, N., 2013. Identification and quantification of bioactive compounds in coffee brews by HPLC–DAD–MSn. Jour*nal of Food Composition and Analysis*, 32(2), 105-115.
Rosiaux, Y., Jannin, V., Hughes, S., Marchaud, D., 2015. Solid lipid excipients as matrix agents for sustained drug delivery. *Excipient Applications in Formulation Design and Drug Delivery*, 237-271.
Rowe, R.C., Sheskey, P., Quinn, M., 2009. Handbook of pharmaceutical excipients. Libros Digitales. Sixth ed. Pharmaceutical Press, Washington. ISBN 978 1 58212 135 2. Diponível em:
Salmela, L., Washington, C., 2014. A continuous flow method for estimation of drug release rates from emulsion formulations. *International Journal of Pharmaceutics*, 472(1-2), 276-281.
Santos, F.L., Nantes, J.F.D., 2014. Coordenação no mercado do café brasileiro: o desserviço da classificação por defeitos. *Gestão & Produção*, 21, 586-599.
Sarkar, R., Podder, I., Gokhale, N., Jagadeesan, S., Garg, V.K., 2017. Use of vegetable oils in dermatology: an overview. *International Journal of Dermatology*, 56(11), 1080-1086.
Scott, S.E., Rozin, P., Small, D.A., 2020. Consumers prefer "natural" more for preventatives than for curatives. *Journal of Consumer Research*, 47(3), 454-471.
Shamsudin, R., Ling, C.S., Adzahan, N.M., Daud, W.R.W., 2013. Rheological properties of ultraviolet-irradiated and thermally pasteurized Yankee pineapple juice. *Journal of Food Engineering*, 116(2), 548-553.
Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In Methods in enzymology (Vol. 299, pp. 152-178). Academic press.
Somnuk, K., Eawlex, P., Prateepchaikul, G., 2017. Optimization of coffee oil extraction from spent coffee grounds using four solvents and prototype-scale extraction using circulation process. *Agriculture and Natural Resources*, 51(3), 181-189.
Speer, K., Kölling-Speer, I., 2006. The lipid fraction of the coffee bean. *Brazilian Journal of Plant Physiology*, 18(1), 201-216.
Spernath, A., Aserin, A., Sintov, A.C., Garti, N., 2008. Phosphatidylcholine embedded micellar systems: Enhanced permeability through rat skin. *Journal of Colloid and Interface Science*, 318(2), 421-429.
Taghvaei, M., Jafari, S.M., 2015. Application and stability of natural antioxidants in edible oils in order to substitute synthetic additives. *Journal of Food Science and Technology*, 52(3), 1272-1282.
Toci, A.T., Farah, A., 2014. Volatile fingerprint of Brazilian defective coffee seeds: Corroboration of potential marker compounds and identification of new low quality indicators. *Food Chemistry*, 153, 298-314.
Tsukui, A., Júnior, H.S., Oigman, S.S., De Souza, R.O.M.A., Bizzo, H.R., Rezende, C.M., 2014. Microwave-assisted extraction of green coffee oil and quantification of diterpenes by HPLC. *Food Chemistry*, 164, 266-271.
Wagemaker, T.A.L., Carvalho, C.R.L., Maia, N.B., Baggio, S.R., Guerreiro Filho, O., 2011. Sun protection factor, content and composition of lipid fraction of green coffee beans. *Industrial Crops and Products*, 33(2), 469-473.
Wagemaker, T.A.L., Fernandes, A.S., Campos, P.M., Rodrigues, L.M., Rijo, P., 2012. Evaluation of antioxidant and antimicrobial activities of green coffee oil in cosmetic formulations. *Biomedical and Biopharmaceutical Research*, 2(9), 207-214.
Wagemaker, T.A.L., Fernandes, A.S., Rosado, C., Rijo, P., Campos, P.M., Rodrigues, L.M., 2013. Safety of green coffee oil in cosmetic formulations: from in vitro to clinical studies. *Toxicology Letters*, (221), S231.
Wagemaker, T.A.L., Silva, S.A., Leonardi, G.R., Campos, P.M.M., 2015. Green *Coffea arabica* L. seed oil influences the stability and protective effects of topical formulations. *Industrial Crops and Products*, 63, 34-40.
Wei, F., Tanokura, M., 2015. Organic compounds in green coffee beans. In Coffee in health and disease prevention. Academic Press, Cambridge, pp. 149-162.
Xu, H., Wang, W., Liu, X., Yuan, F., Gao, Y., 2015. Antioxidative phenolics obtained from spent coffee grounds (*Coffea arabica* L.) by subcritical water extraction. *Industrial Crops and Products*, 76, 946-954.
Zielińska, A., Nowak, I., 2017. Abundance of active ingredients in sea-buckthorn oil. *Lipids in Health and Disease*, 16(1), 1-11.

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REVIEW

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Nyctanthes arbor-tristis L.: Perspective of phytochemical-based inhibition of fatty acid biosynthesis in *Mycobacterium tuberculosis*

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HMG-CoA reductase

ABSTRACT

Nyctanthes arbor-tristis L. contains various phytochemicals with tremendous potential to fight against different infections. However, the effect of these phytochemicals on *Mycobacterium tuberculosis* is yet unknown. Treatment of multi-drug resistance (MDR) and extensively drug-resistant (XDR) strains of the tuberculosis bacterium are still challenging. Therefore, there is an urgent need to overcome this problem. The present review focuses on the potential action of the hypolipidemic phytochemicals obtained from *N. arbor-tristis* on the growth and survival of *M. tuberculosis* in the human host. The extracts from different parts of this plant are hypolipidemic by various established mechanisms. Phytochemicals like iridoids and flavonoids from plant origin exhibit a high capacity to regulate cholesterol and fatty acid biosynthesis *in vivo*. The hypolipidemic properties of *N. arbor-tristis*-derived extracts are probably due to the presence of phytochemicals such as iridoids, flavonoids, etc. It may regulate fatty acid biosynthesis in *M. tuberculosis* by targeting bacterial fatty acid synthase enzyme. Additionally, these phytochemicals also inhibit cholesterol biosynthesis in the host by interrupting the function of HMG-CoA reductase. *M. tuberculosis* is an intracellular pathogen. It is also established fact as on date that entry of tuberculosis bacterium in the macrophage is macrophage membrane cholesterol-dependent. Host cholesterol is also otherwise necessary by multiple mechanisms for the pathogenesis of tuberculosis. Based on the above facts, we believe that *N. arbor-tristis* derived phytochemicals can act both on the tuberculosis bacterium and on the host for prevention and cure of tuberculosis.

1. Introduction

The management of tuberculosis has become far more complex due to the emergence of drug-resistant strains of *Mycobacterium tuberculosis*. In 2020, estimated new tuberculosis cases were around 10 million, while 1.5 million people are died due to the pathogen (WHO, 2021). The current treatment strategy against *M. tuberculosis* includes isoniazid, rifampin, ethambutol, pyrazinamide. It is shown that multidrug-resistant tuberculosis (MDR-TB) is developed due to the resistance of commonly used antimycobacterial agents (Chang et al., 2020; Kim et al., 2019; Sullivan and Amor, 2016). Even extensively drug-resistant (XDR-TB) strains of *M. tuberculosis* are becoming a global problem (Manjelievskaia et al., 2016). Therefore, there is an urgent need to evaluate new or alternative therapeutics to manage tuberculosis patients.

Medicinal plants and herbal products are used to prepare several life-saving medicines. In traditional systems of medicine, various plants are still in use for therapeutic purposes. In the Unani System of medicine, *Nyctanthes arbor-tristis* L. is used as therapeutic (Ahmed et al., 2015). Extracts prepared from different medicinal plants have shown antimycobacterial effects (Gupta et al., 2010). *N. arbor-tristis* extracts have shown antibiotic properties against several microorganisms (Khatune et al., 2001).

The flower extracts obtained from *N. arbor-tristis* exhibits hypolipidemic activity *in vivo* (Rangika et al., 2015). Some phytochemicals present in the plant must be responsible for such hypolipidemic property in the host. Phytochemicals like iridoid glycosides and flavonoids are found extensively in different plant parts of *N. arbor-tristis* (Rathore et al., 1990). Iridoids are secondary metabolites synthesized by various plants, which can be found as

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intermediates in alkaloids synthesis (Dinda et al., 2007). Iridoids like plumericin and isoplumericin, isolated from *Plumeria* (a flowering tree), have a tremendous role in inhibiting mycobacterial growth (Kumar et al., 2013). Extracts from various parts of *N. arbor-tristis* like seeds, leaves, flowers, bark, and fruits consist of different phytochemicals, namely flavonol glycoside, oleanolic acid, essential oils, tannic acid, carotene, friedeline, lupeol, glucose, and benzoic acid (Sah and Verma, 2012). *N. arbor-tristis* extracts have already been recognized as hepatoprotective agents. It has antileishmanial, antiviral, antifungal, antipyretic, antihistaminic, antimalarial, antibacterial, anti-inflammatory, and antioxidant properties as well (Sah and Verma, 2012; Chaudhary et al., 2018). Moreover, major iridoid glycosides like arbortristoside A, B, C, and 6β-hydroxyloganin have also been found in *N. arbor-tristis,* and these iridoids are shown to be effective as antiviral and antileishmanial agents (Gupta et al., 2005; Tandon et al., 1991).

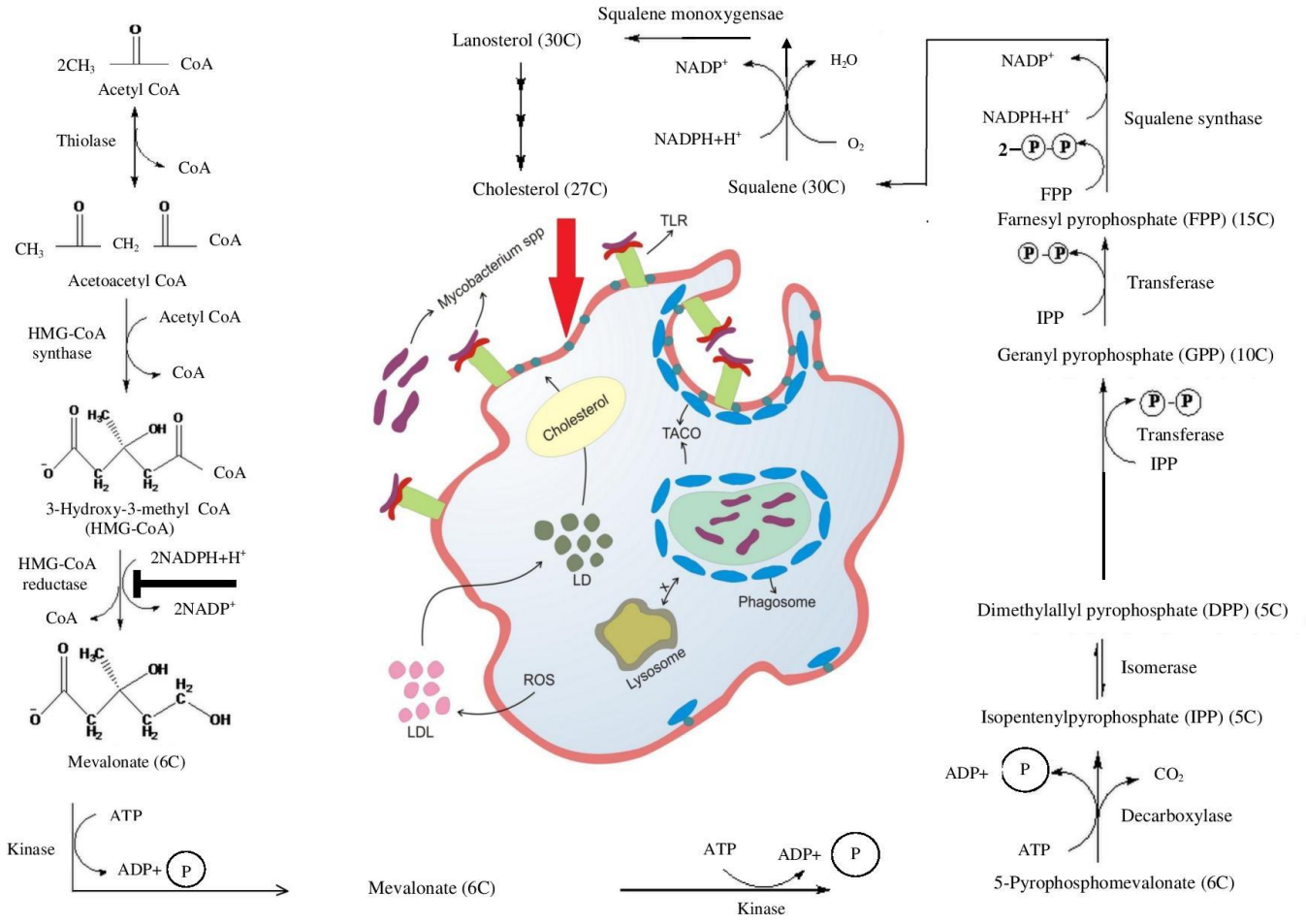

Figure 1. Schematic diagram showing the important enzymatic reactions involved in the intracellular persistence and pathogenesis of *M. tuberculosis* inside host macrophage

The synthesis of cholesterol facilitates the entry and uptake of *M. tuberculosis* inside the macrophage. Reactive oxygen species (ROS) may oxidize low-density lipoproteins (LDL), which subsequently results in the formation of lipid droplets (LD) inside the macrophage. Additionally, cholesterol helps to arrange TACO proteins around the phagosome and prevents phagolysosome complex formation. This mechanism subsequently facilitates the survival and persistence of *M. tuberculosis* inside the macrophage.*N. arbor-tristis* phytochemicals may inhibit the function of HMG-CoA reductase, which ultimately interrupts the bacterial survival and persistence. "⊥" sign represents the inhibitory action of *N. arbor-tristis* phytochemicals.

Iridoids are proved to inhibit fatty acid biosynthesis. Iridoid-glycoside (e.g., ipolamiide) can inhibit type II fatty acid synthase acting through FabI (enoyl acyl carrier protein ACP reductase or enoyl ACP reductase) in *Plasmodium falciparum* (Kirmizibekmez et al., 2004). *M. tuberculosis* uses fatty acid synthase, an essential enzyme for fatty acid biosynthesis and a sole carbon source (Muñoz-Elías and McKinney, 2005). Fatty acid biosynthesis leads to produce propenyl CoA and mycolic acid, which in turn help to compose the cell wall of *M. tuberculosis* (Lee et al., 2013; Korf et al., 2005). Therefore, we feel that iridoids like arbortristoside A, B, C, and 6β-hydroxyloganin (or some other iridoids) present in *N. arbor-tristis* may also reduce the growth of *M. tuberculosis* by interrupting fatty acid synthase activity acting through FabI inhibition. In this context, we wish to emphasize that host fatty acids can also modulate the pathogenesis of tuberculosis (Russell, 2003). So, if iridoids inhibit host fatty acid biosynthesis, that will be an attractive mechanism for controlling tuberculosis. However, as of date, there is evidence that *N. arbor-tristis*-derived products reduce triglyceride in the host (Rathod et al., 2009; Rangika et al., 2015). This reduction of triglycerides is due to the control of insulin sensitivity or direct inhibition of fatty acid biosynthesis in the host is not very clear as on date. Keeping this controversy alive, it can be logically concluded that the plant products can control tuberculosis because diabetes mellitus is also a risk factor for tuberculosis (Dooley and Chaisson, 2009). So, control of diabetes mellitus by the products of *N. arbor-tristis* as reported in various literature, may improve the tuberculosis status at least in a diabetic host (Rangika et al., 2015; Kul et al., 2015).

Additionally, cholesterol biosynthesis plays a significant role in the intracellular persistence of *M. tuberculosis* (Miner et al., 2009).

Therefore, inhibiting cholesterol synthesis might be an alternative strategy to inhibit *M. tuberculosis*. Several plant-derived phytochemicals have been shown to inhibit cholesterol synthesis by inhibiting HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase) or degrading lipid moieties (Middleton and Kok-Pheng, 1982; Reddy Palvai and Urooj, 2014; Palu et al., 2012). Hence, it is logical to think that iridoid glycosides, extracted from *N. arbor-tristis*, may decrease the survival of *M. tuberculosis* by reducing cholesterol biosynthesis through inhibition of HMG-CoA reductase. Apart from iridoids, several flavonoids like nicotiflorin, phenylpropanoid, naringenin-4'-*O*-β-D-glucopyranosyl-α-xylopyranoside, astragalin, etc. are found in *N. arbor-tristis*. Flavonoids from leaf extract of various plants are shown to exert a high degree of fatty acid synthase inhibition (Chen et al., 2009). On the other hand, flavonoids can lower cholesterol levels by inhibiting the function of HMG-CoA reductase (Baskaran et al., 2015). Hence, we believe that flavonoids present in *N. arbor-tristis* may reduce the growth and survival of *M. tuberculosis* by inhibiting HMG-CoA reductase. Critical ethnopharmacological review is already available for the plant concerned (Agrawal and Pal, 2013). However, from a mechanistic point of view, we feel that phytochemicals obtained from *N. arbor-tristis* have significant potential for preventing and curing tuberculosis, and this aspect is reviewed here. We consider that such a review of basic biology and ethnopharmacological relevance related to the context is necessary for novel drug discovery to prevent and cure tuberculosis.

2. Methodology

A thorough literature survey has been done to review the studies published so far in the concerned field available freely on public domains such as Pubmed and Google Scholar. Original studies, review articles, and books are referred to as search articles related to the present review article from 1977 to 2021. "*Nyctanthes arbor-tristis* L." as a keyword is predominantly used to cite reference articles. However, other keywords such as "*Mycobacterium tuberculosis*", iridoid, cholesterol, fatty acid, HMG-CoA reductase are also associated with *N. arbor-tristis*. Only peer-reviewed publications related to the keywords mentioned above have been included in this article. In contrast, articles published in other languages except English are excluded.

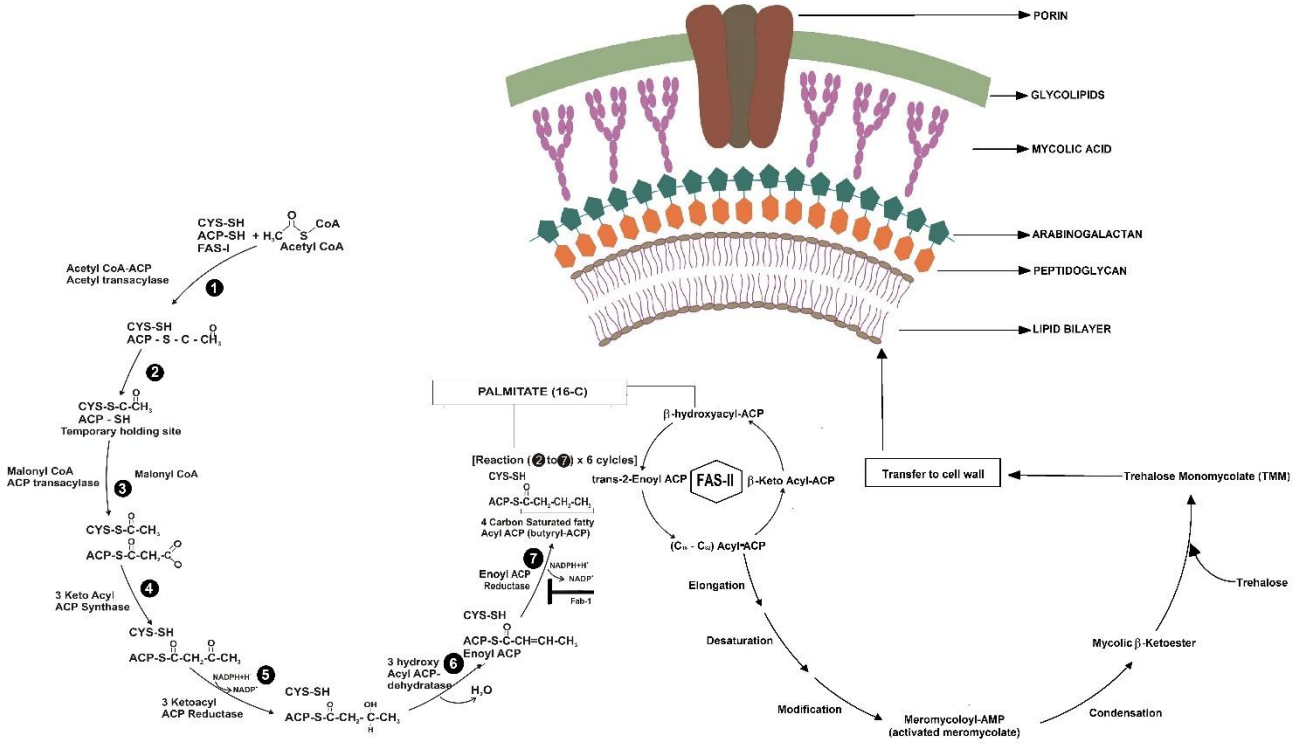

Figure 2. Schematic diagram representing the probable enzymatic reactions mediated combinedly by FAS-I and FAS-II by *M. tuberculosis* to synthesize long-chain fatty acyl ester

N. arbor-tristis phytochemicals may inhibit the function of Enoyl ACP reductase, which ultimately results in the inhibition of *M. tuberculosis* cell wall synthesis. "⊥" sign represents the inhibitory action of *N. arbor-tristis* phytochemicals.

3. The role of cholesterol in the pathogenesis of *M. tuberculosis*

Cholesterol is an essential constituent of the plasma membrane, which provides fluidity and permeability (Ikonen, 2008). Additionally, cholesterol acts as a vital precursor for synthesizing bile salts, steroid hormones, and vitamin D. Animals readily synthesize cholesterol. Even fungi and plants are also capable of producing cholesterol. However, sterol biosynthesis is still contentious in bacteria (Fuerst and Sagulenko, 2014). *M. tuberculosis* lacks cholesterol biosynthesis machinery like monooxygenase etc. (Podust et al., 2004). Therefore, the "smart bacteria" *M. tuberculosis* uses the host cholesterol to maintain its pathogenesis and intracellular persistence (Figure 1). Interestingly, host cholesterol plays as a triggering factor in tuberculosis development (Miner et al., 2009). Even extra consumption of dietary cholesterol may influence the virulence mechanism of *M. tuberculosis* (Schafer et al., 2009). Besides, cholesterol is required to anchor host protein coronin 1 or tryptophan-aspartate-containing coat protein (TACO) on the surface of the phagosome. TACO inhibits the phagosome-lysosome fusion during infection (Anand and Kaul,

2005). Hence, inhibiting cholesterol biosynthesis may be a step forward to control mycobacterial survival. It is particularly relevant in the present context since *M. tuberculosis* enters the host macrophage through the cholesterol-rich region of the macrophage membrane (Gatfield and Pieters, 2000).

4. Fatty acid synthase and mycobacterial growth

Fatty acid synthase (E.C. 2.3.1.85) is an essential enzyme for *M. tuberculosis* to synthesize long-chain fatty acid through sequential condensation of acetyl-coenzyme A (acetyl-CoA) and malonyl-CoA (Ngo et al., 2007). This enzyme needs to produce the major cell wall components like mycolic acid etc. (Figure 2). Fatty acid biosynthesis is crucial for *M. tuberculosis* for its intracellular persistence because, in the presence of host-derived fatty acid, this bacteria is survived by isocitrate lyase of glyoxylate cycle (Gould et al., 2006). The complex cell wall structure contributes to less permeability and fluidity. This helps *M. tuberculosis* to develop resistance towards multiple drugs when surviving in a protected phagosome that does not fuse with the lysosome (Ciccarelli et al., 2013). Therefore, new drugs may be designed to target bacterial cell wall synthesis. Mycobacterial fatty acid biosynthesis mainly depends on type I (FAS I) and type II (FAS II) fatty acid synthase. FAS I is present in mammalian and higher eukaryotes, whereas FAS II is present only in prokaryotes. The complete enzymatic process of mycobacterial fatty acid biosynthesis is poorly understood as of date due to the slow growth of the bacterium in the available culture systems. However, it is known beyond any doubt that both FAS I and FAS II systems are essential for the synthesis of C80 mycolic acid. Initially, FAS I synthesizes C16-C26 acyl-CoA esters, ultimately producing C80 mycolic acid by FAS II (Cole et al., 1998).

5. Inhibition of long-chain fatty acid biosynthesis as a measure for controlling tuberculosis

As mentioned earlier, fatty acid biosynthesis is necessary to synthesize major cell wall components, thus crucial for bacterial survival and drug resistance. Several studies have shown the inhibition of this bacterium by either targeting fatty acid synthase or mycolic acid formation. Ngo et al. (2007) have shown that pyrazinamide (PZA) and its analog of 5-chloropyrazinamide (5-Cl-PZA) can inhibit FAS I in *M. smegmatis*. Moreover, 2-hexadecynoic acid and 2-octadecynoic acid show bactericidal effects against *M. smegmatis* and *M. bovis*. Having higher concentrations, 2-octadecynoic acid accumulates 3-ketohexadecanoic acid, which further inhibits fatty acid biosynthesis and formation of mycolic acid (Morbidoni et al., 2006). Isoxyl and thiacetazone cause the accumulation of 3-hydroxy C18, C20, and C22 fatty acids, which ultimately inhibit the dehydratase step of the fatty acid synthase type II-mediated elongation (Grzegorzewicz et al., 2012a). Besides, adamantyl urea-based compound is also being employed successfully to inhibit mycolic acid transport across the plasma membrane (Grzegorzewicz et al., 2012b).

6. Recent approaches to inhibit cholesterol biosynthesis in tuberculosis

The persistence of *M. tuberculosis* mainly depends on utilizing the host cholesterol (Pandey and Sassetti, 2008). Apart from this, the cellular lipids play a pivotal role in the progression towards the latent stage (Ahmad et al., 2006; Ouellet et al., 2011). So, the inhibition of cholesterol synthesis may affect the survival of the bacteria. Recent strategies comprise a classical mechanism to inhibit cholesterol synthesis, which is based on the inhibition of HMG-CoA reductase (Parihar et al., 2014). HMG-CoA reductase is an essential enzyme that catalyzes HMG-CoA conversion to mevalonate in cholesterol biosynthesis (Endo, 2010) (Figure 1). In the year 1977, it has been first reported that citrinin can reduce cholesterol levels by inhibiting HMG-CoA reductase (Kazuhiko et al., 1977). In broadway, cholesterol-lowering drugs can be classified into two major groups-statins (e.g., lovastatin, atorvastatin, etc.) and fibrates (e.g., ciprofibrate, bezafibrate, etc.) (Pahan, 2006). The mechanism of statin is based on the competitive inhibition of HMG-CoA reductase, whereas fibrates stimulate β-oxidation of fatty acids in peroxisomes and mitochondria. Moreover, statins are hypothesized to treat *M. tuberculosis* as it potentially inhibits HMG-CoA reductase (Parihar et al., 2014). However, these drugs are still not safe due to their toxic side effects (Endo, 2010; Golomb and Evans, 2008). Therefore, alternative approaches are required, which would have less toxicity.

7. Medicinal plant extracts against *M. tuberculosis*

Medicinal plants have a significant role in controlling the growth of *M. tuberculosis*. Extracts from different parts of plants are used to treat *M. tuberculosis,* and among them, the leaf extract is supposed to be more active (Gupta et al., 2010). Leaf extract from *Allium cepa* L., *A. sativum* L., and *Aloe vera* L. Burm. F. have shown antitubercular activity up to 68% against H37Rv strain (Gupta et al., 2010). Extracts prepared from various plants are proved to be bactericidal for several mycobacterial species, including MDR strains of *M. tuberculosis* (Bueno-Sánchez et al., 2009; Lawal et al., 2014; Mariita et al., 2010; Sheeba et al., 2015; Shukla and Sharma, 2021; Sivakumar and Jayaraman, 2011).

8. Inhibition of cholesterol biosynthesis by different herbal extracts and phytochemicals

As mentioned earlier, cholesterol biosynthesis plays a crucial role in the intracellular survival of *M. tuberculosis*. In this stage, the bacterium uses the host cholesterol from the plasma membrane and accumulates the lipid droplets into the macrophage (Daniel et al., 2011). It is now proved that many medicinal plants contain phytochemicals that inhibit cholesterol biosynthesis. The leaf extract of *Basella alba* L. has phytochemicals such as α-tocopherol, oleic acid, apigenin, luteolin, ascorbic acid, naringin, and eicosyl ester are reported to inhibit HMG-CoA reductase *in vivo* (Baskaran et al., 2015). Additionally, iridoids and precursors like picroliv, oleuropein, geraniol, and limonene can act as hypolipidemic agents (Khanna et al., 1994; Omar, 2010; Pattanayak et al., 2009). While, monoterpene like rowachol successfully inhibits HMG-CoA reductase in the rat model (Middleton and Kok-Pheng, 1982). *Morinda citrifolia* L. extracts alone or in combination with iridoid are shown to inhibit HMG-CoA reductase (Palu et al., 2012; West et al., 2014).

On the other hand, flavonoids are the secondary metabolites of plants, which are synthesized through the phenylpropanoid pathway (Falcone Ferreyra et al., 2012). Flavonoids from plant origin have shown tremendous cholesterol-lowering effects *in vivo* (Esmaillzadeh et al., 2006; Miyake et al., 2006; Roza et al., 2007). Flavonoids obtained from *B. alba* leaf extract exert the inhibition of HMG-CoA reductase activity (Baskaran et al., 2015).

9. Antimycobacterial effects of *N. arbor-tristis*

So far, we have illustrated the importance of different medicinal plants and phytochemicals having antimycobacterial activity, cholesterol-lowering ability, and inhibitory role in fatty acid biosynthesis. There is evidence regarding the medicinal effect of extracts from different parts of *N. arbor-tristis* in various infectious diseases (Rahman et al., 2013; Murti et al., 2012) as well as in

metabolic disorders (Agrawal and Pal, 2013; Khatune et al., 2001) (Figure 3). Additionally, the flower extract exhibits hypolipidemic activity in the mice model (Rangika et al., 2015). The antimycobacterial effect and hypolipidemic characteristics of the plant extract may be due to the presence of different phytochemicals. The two most important classes of phytochemicals found in *N. arbor-tristis* are iridoid glycosides (e.g., arbortristoside-A, B, C, 6β-hydroxyloganin, etc.) and flavonoids (e.g., nicotiflorin, phenylpropanoid, naringenin-4'-*O*-β-D-glucopyranosyl-α-xylopyranoside, astragalin, etc.) (Table 1). Unlike the other iridoids, monoterpenes such as loganins are produced from the non-mevalonate pathway (Eichinger et al., 1999). Loganin glycoside extracted from plant sources can act as a hypolipidemic substance by regulating PI3K–Akt/PKB signaling pathway (Yamabe et al., 2010; Kang et al., 2018). In context, 6β-hydroxyloganin obtained from *N. arbor-tristis* is reported as anti-leishmania and anti-arthritic agents (Tandon et al., 1991).

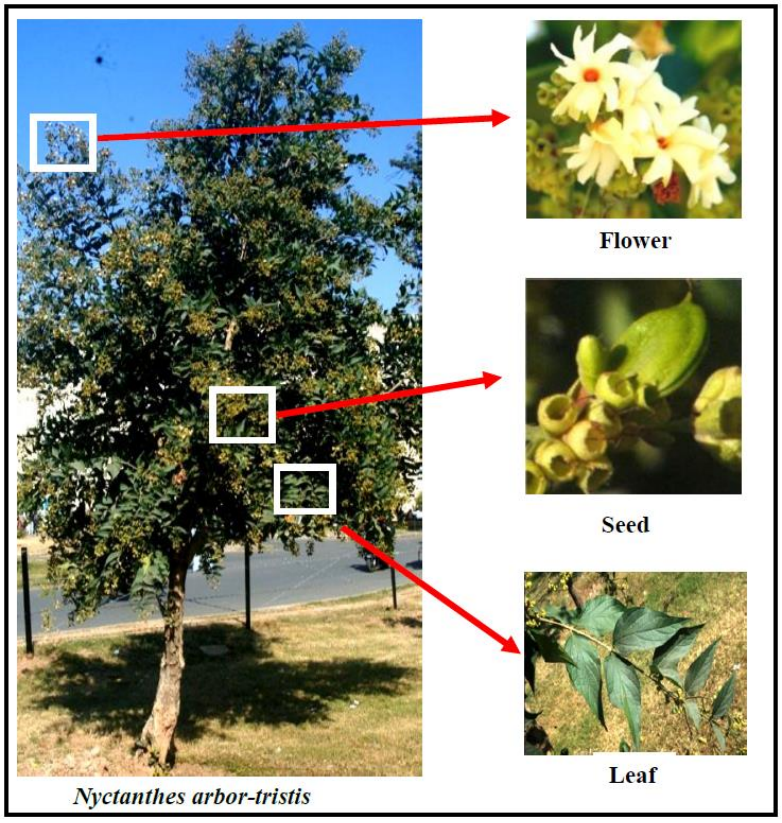

Figure 3. Photograph showing *N. arbor-tristis* tree and its different parts such as flower, seed, and leaf

For a long time, medicinal plants have been used in the Indian traditional "Ayurveda" to treat different infectious diseases, including pulmonary tuberculosis (Debnath et al., 2012). As mentioned earlier, different herbs exhibited antitubercular effects against specific mycobacterial species, including drug-resistant strains. Besides, plant phytochemicals like iridoid glycosides, flavonoids, terpenoids are capable of inhibiting the growth of *M. tuberculosis* by an unknown mechanism and are reported as a novel antituberculosis drug (Askun et al., 2013; Cantrell et al., 2001; Gordien et al., 2009; Jimenez-Arellanes et al., 2013; Kumar et al., 2013; Zheng et al., 2014). These phytochemicals have different intracellular targets with potential for cholesterol biosynthesis inhibition, fatty acid synthase inhibition, etc. (Duan et al., 2012; Khanna et al., 1994; Kirmizibekmez et al., 2004; Li et al., 2014; Sundaram et al., 2013; Tasdemir et al., 2005; Wang et al., 2012; Yamabe et al., 2007).

Iridoids obtained from plant sources show the potential therapeutic role against trypanosomiasis and leishmaniasis parasites. Iridoid-rich plant extracts can cease fatty acid biosynthesis in *Plasmodium falciparum* by inhibiting FabI enzyme activity (Tasdemir et al., 2005). *M. tuberculosis* is an intracellular pathogen like *P. falciparum*, *Trypanosoma bruceirhodesiense*, *Leishmania donovani*; therefore, we believe that the iridoid glycosides like arbortristosides A, B, C, etc. obtained from the extract of *N. arbor-tristis* may also exert the inhibitory role against *M. tuberculosis* by analogous mechanism.

10. Hypothetical interventions

M. tuberculosis has a tremendous capacity to use the host machinery for their survival and intracellular persistence. One of the important mechanisms of *M. tuberculosis* pathogenesis is based on the utilization of host cholesterol (Ahmad et al., 2006; Ouellet et al., 2011; Pandey and Sassetti, 2008). Further, fatty acid biosynthesis is crucial for the bacterium to produce cell wall components like mycolic acid (Takayama et al., 2005). The importance of cholesterol biosynthesis inhibition is believed to be an important method for preventing and curing tuberculosis (Parihar et al., 2014). Cholesterol biosynthesis inhibition also increases the bacteriocidal effects of known antimycobacterial drugs (Lobato et al., 2014). Inhibition of bacterium fatty acid biosynthesis, particularly mycolic acid biosynthesis, is caused by established antimycobacterial drugs (Schroeder et al., 2002).

Considering the above facts, we believe that extracts of *N. arbor-tristis* may cause cholesterol biosynthesis inhibition in the host and fatty acid biosynthesis inhibition in *M. tuberculosis*. We feel so because *N. arbor-tristis* is rich in phytochemicals like iridoids,

flavonoids, etc. (Table 1) (Sah and Verma, 2012). It is also proved beyond any doubt that the phytochemicals mentioned above have lipid-lowering action in the host (Murti et al., 2012; Rathod et al., 2009). Crude extracts prepared from the flower and the root of *N. arbor-tristis* have already been reported to reduce cholesterol levels in the mice model (Rangika et al., 2015). Phytochemicals in *N. arbor-tristis* (e.g., iridoids, Aglycone Ag-NY1) can inhibit host cholesterol biosynthesis by inhibiting HMG-CoA reductase, the key enzyme for cholesterol biosynthesis. Therefore, *N. arbor-tristis*-derived phytochemicals can control the growth and survival of *M. tuberculosis* in human hosts by reducing host cholesterol. Some phytochemicals derived from *N. arbor-tristis* (e.g., arbortristoside A, B, C) may inhibit the fatty acid biosynthesis of *M. tuberculosis* by targeting fatty acid synthase (FAS II). In this context, flavonoids present in N. arbor-tristis deserve a special mention because flavonoids are known to inhibit the growth of *M. tuberculosis* by inhibiting bacterial fatty acid biosynthesis (Brown et al., 2007). Therefore, phytochemicals derived from the plant can target bacterial mycolic acid biosynthesis and exert bacteriocidal action. In simple words, we feel that *N. arbor-tristis* derived phytochemicals can be beneficial for tuberculosis by reducing host cholesterol as well as by inhibiting bacterial mycolic acid biosynthesis. This is a unique approach as far as our knowledge goes. The present methodology of tuberculosis control either works through the host immune system (Bacille Calmette-Guerin vaccine) or the bacteria (INH, Rifampicin, etc.). However, *N. arbor-tristis* derived phytochemicals can prevent and cure tuberculosis, acting both through the host and the bacteria. Additionally, the extracts of *N. arbor-tristis* are known for controlling hyperglycemia. Since diabetes mellitus is an established risk factor for tuberculosis, controlling glycemia in diabetic patients will also help prevent tuberculosis by the plant products. However, future *in vitro* and *in vivo* studies based on our hypothesis may be a step forward to take alternative and safe therapeutic strategies for managing tuberculosis patients.

Table 1. List of phytochemicals present in the *N. arbor-tristis* plant and their functions to inhibit fatty acid and cholesterol biosynthesis

No	Phytochemicals			Inhibitory roles in fatty acid/cholesterol biosynthesis	References
	Name	Source/Plant parts	Chemical class		
1	Arbortristoside A	Seed	Iridoid	Cholesterol and triglycerides lowering effect	Rangika et al., 2015; Rathod et al., 2009; Murti et al., 2012; Rani et al., 2012
2	Arbortristoside B	Seed	Iridoid	Cholesterol and triglycerides lowering effect	Rangika et al., 2015; Rathod et al., 2009; Murti et al., 2012; Rani et al., 2012
3	Arbortristoside C	Seed	Iridoid	Cholesterol and triglycerides lowering effect	Rangika et al., 2015; Rathod et al., 2009; Murti et al., 2012; Rani et al., 2012
4	Arbortristoside D	Seed	Iridoid	Not reported	-
5	Arbortristoside E	Seed	Iridoid	Not reported	-
6	Glycerides	Seed	Ester	Not reported	-
7	Lignoceric acid	Seed	Saturated fatty acid	Not reported	-
8	Stearic acid	Seed, bark	Saturated fatty acid	Reduce LDL cholesterol	Mensink, 2005
9	Palmitic acid	Seed, bark	Saturated fatty acid	Inhibitor of fatty acid synthesis in mammary tissue	Wright et al., 2002
10	Myristic acid	Seed, bark	Saturated fatty acid	Not reported	-
11	3-4 Secotriterpene acid	Seed	Amide conjugate	Not reported	-
12	D-Glucose	Seed, root	Carbohydrate	Not reported	-
13	D-Mannose	Seed	Carbohydrate	Not reported	-
14	Friedelin	Seed	Terpenoid	Not reported	-
15	Nycanthin	Flower	Terpenoid	Not reported	-
16	Flavonoids	Flower	Flavonoid	Inhibition of *in vitro* cholesterol synthesis, beta-ketoacyl synthase domain of FAS to inhibit the elongation of the saturated acyl groups in fatty acids synthesis.	Brown et al., 2007
17	Anthocyanins	Flower	Flavonoids	Not reported	-
18	Essential oil	Flower	Fatty acid	Not reported	-
19	β-Monogentiobioside	Flower	Carotenoid	Not reported	-
20	β-Digentiobioside	Flower	Carotenoid	Not reported	-
21	α-Pinene	Flower	Terpenoid	Inhibition of fatty acid synthesis	Aydin et al., 2013
22	*p*-Cymene	Flower	Terpenoid	Not reported	-
23	1-hexanol methyl heptanone	Flower	Ketone	Not reported	-
24	Phenylacetaldehyde	Flower	Aldehyde	Not reported	-
25	1-Deconol	Flower	Fatty alcohol	Not reported	-
26	Anisaldehyde	Flower	Aldehyde	Not reported	-
27	4-hydroxy hexahydrobezofuran-7-one	Flower, leaf	Flavonoids	Not reported	-
28	Rengyolone and its acetate NCS-2	Flower	Flavonoids	Not reported	-
29	Aglycone Ag-NY1	Tubular calyx of flowers	Carotenoid	Inhibit both the effect of HMG CoA reductase and acyl CoA: cholesterol *O*-acyltransferase activities.	Gebhardt, 1998
30	6β-Hydroxyloganin	Leaf	Iridoid	Not reported	-
31	D-Mannitol	Leaf, root	Carbohydrate	Not reported	-
32	β-Sitosterol	Leaf	Phytosterol	Not reported	-
33	Astragalin	Leaf	Flavonoid	Inhibit cholesterol synthesis in higher concentration	-
34	Nicotiflorin	Leaf	Flavonoid	Not reported	-
35	Oleanolic acid	Leaf	Terpenoid	Inhibit the fat production	-
36	Nyctanthic acid	Leaf	Terpenoid	Not reported	-
37	Tannic acid	Leaf	Polyphenol	Reduce total and LDL cholesterol in cholesterol-fed rats. Inhibit fatty acid synthase activity.	-
38	Ascorbic acid	Leaf	Vitamin	High concentrations of vitamin C inhibit the activity of HMG-CoA reductase *in vitro*.	Harwood et al., 1986
39	Methyl salicylate	Leaf	Glucoside	Not reported	-

No	Phytochemicals			Inhibitory roles in fatty acid/cholesterol biosynthesis	References
	Name	Source/Plant parts	Chemical class		
40	Volatile oil	Leaf	Fatty acid	Not reported	-
41	Friedeline	Leaf	Terpenoid	Lowering of lipid levels in plasma and liver	Duraipandiyan et al., 2016
42	Lupeol	Leaf	Terpenoid	Lupeol reduces triglyceride and cholesterol synthesis in human hepatoma cells	Itoh et al., 2009
43	Glucose	Leaf	Carbohydrate	Not reported	-
44	Calceolarioside A	Leaf	Verbascoside	Not reported	-
45	Naringenin-4'-*O*-β-D-glucopyranosyl-α-xylopyranoside	Stem	Flavonoid	Not reported	-
46	β-sitosterol	Stem	Phytosterols	Not reported	-
47	Iridoid	Bark	Terpenoid	Monoterpenes have been found to inhibit hepatic HMG CoA reductase by 50 to 60%	Middleton and Kok-Pheng, 1982
48	Phenylpropanoid	Bark, seed	Flavonoid	Inhibition of triglyceride (TG) synthesis and promotion of cholesterol catabolism.	Yang et al., 2015
49	Tannin	Root	Polyphenol	A low concentration of condensed tannins from catechu significantly inhibits fatty acid synthase.	Zhang et al., 2008
50	Caproic acid	Bark	Fatty acid	-	-
51	Nonanoic acid	Bark	Fatty acid	-	-
52	Capric acid	Bark	Fatty acid	-	-
53	Undecanoic acid	Bark	Fatty acid	-	-
54	Lauric acid	Bark	Fatty acid	-	-
56	Tridecanoic acid	Bark	Fatty acid	-	-
57	Myristoleic acid	Bark	Fatty acid	-	-
58	Pentadecanoic acid	Bark	Fatty acid	-	-
59	Palmitoleic acid	Bark	Fatty acid	-	-
60	Heptadecanoic acid	Bark	Fatty acid	-	-
61	Oleic acid	Bark	Fatty acid	-	-
62	Linoleic acid	Bark	Fatty acid	-	-
63	α-Linolenic acid	Bark	Fatty acid	-	-
64	Arachidic acid	Bark	Fatty acid	-	-
65	Eicosenoic acid	Bark	Fatty acid	-	-
66	Octadecatetraenoic acid	Bark	Fatty acid	-	-
67	Eicosadienoic acid	Bark	Fatty acid	-	-
68	Arachidonic acid	Bark	Fatty acid	-	-
69	Behenic acid	Bark	Fatty acid	-	-
70	Nyctanthin	Root	Alkaloid	-	-

11. Conclusions

The current treatment of tuberculosis mainly relies on the drugs such as isoniazid, rifampin, ethambutol, pyrazinamide (Yew et al., 2010; McIlleron et al., 2006). The choice of multi drugs is due to reducing both initial bacterial load and persisters. The first-line drug such as isoniazid can reduce the initial bacterial load by about 95% within the first two days (Joshi, 2011). Rifampicin is quite effective against the persisters during the initial failure of isoniazid due to resistant or sensitive strains. The period of these multidrug chemotherapies with a minimum of nine months to a maximum of eighteen months could be reduced to six months by adding pyrazinamide. Due to the failure of the first-line drugs, the second-line treatment could be prescribed for the period of eighteen to twenty-four months of prophylaxis. The extended prophylaxis of TB drugs often develops resistance mechanisms in tubercle bacilli due to certain mutational changes (Veen, 1995). Additionally, prolonged treatment with a higher concentration of TB drugs becomes toxic for internal organs like the liver, kidney, etc. (Saukkonen et al., 2006). Thus, there is a need to improve the therapeutic approaches by employing safe and effective drugs against the pathogenesis of *M. tuberculosis*. The present review indicates a specific and target-based approach against the pathogenesis of *M. tuberculosis*. There is evidence that iridoids, flavonoids, etc., obtained from different sources have potential lipid-lowering action (Khanna et al., 1994). Besides, based on different studies and reports, we believe that major phytochemicals obtained from the plant extract of *N. arbor-tristis* may also exhibit a similar mechanism of action (Debnath et al., 2012; Rangika et al., 2015; Rathod et al., 2009). The lipid-lowering action appears in the host and the bacteria, which is expected to have a profound antimycobacterial role. The anti-ulcerogenic, ulcer-healing properties and *in vivo* studies of these phytochemicals instigate us to further assess them as safe, non-toxic natural products (Ekeanyanwu and Njoku, 2014; Mishra et al., 2013; Rani et al., 2012). However, *in vitro* and in vivo studies are required in a large cohort in this regard. Further studies need to be conducted to explore the molecular interactions between *N. arbor-tristis* phytochemicals and *M. tuberculosis*, which may exhibit the target-specific inhibition of the bacterium. And we feel that validation of our hypothesis is urgently required since the modern world is facing a threat from multidrug-resistant tuberculosis, which demands exploration of novel strategies for effective prevention and control of tuberculosis.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

Subendu Sarkar: Conceptualization, Data curation, Investigation, Writing-reviewing & editing the manuscript, Reviewing the manuscript
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Supplementary File

None.

References

Agrawal, J., Pal, A., 2013. *Nyctanthes arbor-tristis* Linn—A critical ethnopharmacological review. *Journal of Ethnopharmacology*, 146(3), 645-658.

Ahmad, Z., Sharma, S., Khuller, G.K., 2006. The potential of azole antifungals against latent/persistent tuberculosis. *FEMS Microbiology Letters*, 258(2), 200-203.

Ahmed, K.I., Opu, S.A., Muttaki, A.A., Al-Mamun, M., Islam, M.T., Das, P.R., Rahmatullah, M., 2015. Plant remedies of a unani medicinal practitioner in Bhola district, Bangladesh. *World Journal of Pharmacy and Pharmaceutical Sciences*, 4, 186-198.

Anand, P.K., Kaul, D., 2005. Downregulation of TACO gene transcription restricts mycobacterial entry/survival within human macrophages. *FEMS Microbiology Letters*, 250(1), 137-144.

Askun, T., Tekwu, E.M., Satil, F., Modanlioglu, S., Aydeniz, H., 2013. Preliminary antimycobacterial study on selected Turkish plants (Lamiaceae) against *Mycobacterium tuberculosis* and search for some phenolic constituents. *BMC Complementary and Alternative Medicine*, 13(1), 1-11.

Aydin, E., Türkez, H., Geyikoğlu, F., 2013. Antioxidative, anticancer and genotoxic properties of α-pinene on N2a neuroblastoma cells. *Biologia*, 68(5), 1004-1009.

Baskaran, G., Salvamani, S., Ahmad, S.A., Shaharuddin, N.A., Pattiram, P.D., Shukor, M.Y., 2015. HMG-CoA reductase inhibitory activity and phytocomponent investigation of *Basella alba* leaf extract as a treatment for hypercholesterolemia. *Drug Design, Development and Therapy*, 9, 509-517.

Brown, A.K., Papaemmanouil, A., Bhowruth, V., Bhatt, A., Dover, L.G., Besra, G.S., 2007. Flavonoid inhibitors as novel antimycobacterial agents targeting Rv0636, a putative dehydratase enzyme involved in *Mycobacterium tuberculosis* fatty acid synthase II. *Microbiology*, 153(10), 3314-3322.

Bueno-Sánchez, J.G., Martínez-Morales, J.R., Stashenko, E.E., Ribón, W., 2009. Anti-tubercular activity of eleven aromatic and medicinal plants occurring in Colombia. *Biomedica*, 29(1), 51-60.

Cantrell, C.L., Franzblau, S.G., Fischer, N.H., 2001. Antimycobacterial plant terpenoids. *Planta Medica*, 67(08), 685-694.

Chang, K.C., Yew, W.W., 2020. ATS/CDC/ERS/IDSA clinical practice guidelines for treatment of drug-resistant tuberculosis: a two-edged sword?. *American Journal of Respiratory and Critical Care Medicine*, 202(5), 777-778.

Chaudhary, S., Gupta, R.K., Kumar, A., Tarazi, H., 2018. Hepatoprotective and antioxidant potential of *Nyctanthes arbor-tristis* L. leaves against antitubercular drugs induced hepatotoxicity. *Journal of Pharmacy & Pharmacognosy Research*, 6(3), 205-215.

Chen, J., Zhuang, D., Cai, W., Xu, L., Li, E., Wu, Y., Sugiyama, K., 2009. Inhibitory effects of four plants flavonoids extracts on fatty acid synthase. *Journal of Environmental Sciences (China)*, 21, S131-S134.

Ciccarelli, L., Connell, S.R., Enderle, M., Mills, D.J., Vonck, J., Grininger, M., 2013. Structure and conformational variability of the *Mycobacterium tuberculosis* fatty acid synthase multienzyme complex. *Structure*, 21(7), 1251-1257.

Cole, S., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Barrell, B.G., 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 396(6707), 190-190.

Daniel, J., Maamar, H., Deb, C., Sirakova, T.D., Kolattukudy, P.E., 2011. *Mycobacterium tuberculosis* uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS Pathogens*, 7(6), e1002093.

Debnath, P.K., Chattopadhyay, J., Mitra, A., Adhikari, A., Alam, M.S., Bandopadhyay, S.K., Hazra, J., 2012. Adjunct therapy of Ayurvedic medicine with anti tubercular drugs on the therapeutic management of pulmonary tuberculosis. *Journal of Ayurveda and Integrative Medicine*, 3(3), 141-149.

Dinda, B., Debnath, S., Harigaya, Y., 2007. Naturally occurring iridoids. A review, part 1. *Chemical and Pharmaceutical Bulletin*, 55(2), 159-222.

Dooley, K.E., Chaisson, R.E., 2009. Tuberculosis and diabetes mellitus: convergence of two epidemics. *The Lancet Infectious Diseases*, 9(12), 737-746.

Duan, C., Wang, Y., Ma, X., Jiang, Y., Liu, J., Tu, P., 2012. A new furostanol glycoside with fatty acid synthase inhibitory activity from *Ophiopogon japonicusa*. *Chemistry of Natural Compounds*, 48(4), 613-615.

Duraipandiyan, V., Al-Dhabi, N.A., Irudayaraj, S.S., Sunil, C., 2016. Hypolipidemic activity of friedelin isolated from *Azima tetracantha* in hyperlipidemic rats. *Revista Brasileira de Farmacognosia*, 26, 89-93.

Eichinger, D., Bacher, A., Zenk, M.H., Eisenreich, W., 1999. Analysis of metabolic pathways via quantitative prediction of isotope labeling patterns: a retrobiosynthetic 13C NMR study on the monoterpene loganin. *Phytochemistry*, 51(2), 223-236.

Ekeanyanwu, R.C., Njoku, O.U., 2014. Acute and subacute oral toxicity study on the flavonoid rich fraction of *Monodora tenuifolia* seed in albino rats. *Asian Pacific Journal of Tropical Biomedicine*, 4(3), 194-202.

Endo, A., 2010. A historical perspective on the discovery of statins. *Proceedings of the Japan Academy, Series B*, 86(5), 484-493.

Esmaillzadeh, A., Tahbaz, F., Gaieni, I., Alavi-Majd, H., Azadbakht, L., 2006. Cholesterol-lowering effect of concentrated pomegranate juice consumption in type II diabetic patients with hyperlipidemia. *International Journal for Vitamin and Nutrition Research*, 76(3), 147-151.

Falcone Ferreyra, M.L., Rius, S., Casati, P., 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in Plant Science*, 3, 222.

Fuerst, J.A., Sagulenko, E., 2014. Towards understanding the molecular mechanism of the endocytosis-like process in the bacterium *Gemmata obscuriglobus*. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1843(8), 1732-1738.

Gatfield, J., Pieters, J., 2000. Essential role for cholesterol in entry of mycobacteria into macrophages. *Science*, 288(5471), 1647-1651.

Gebhardt, R., 1998. Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (*Cynara scolymus* L.) extracts. *Journal of Pharmacology and Experimental Therapeutics*, 286(3), 1122-1128.

Golomb, B.A., Evans, M.A., 2008. Statin adverse effects: A review of the literature and evidence for a mitochondrial mechanism. *American Journal of Cardiovascular Drugs*, 8(6), 373-418.

Gordien, A.Y., Gray, A.I., Franzblau, S.G., Seidel, V., 2009. Antimycobacterial terpenoids from *Juniperus communis* L.(Cuppressaceae). *Journal of Ethnopharmacology*, 126(3), 500-505.

Gould, T.A., Van De Langemheen, H., Muñoz-Elías, E.J., McKinney, J.D., Sacchettini, J.C., 2006. Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in *Mycobacterium tuberculosis*. *Molecular Microbiology*, 61(4), 940-947.

Grzegorzewicz, A.E., Korduláková, J., Jones, V., Born, S.E., Belardinelli, J.M., Vaquié, A., Jackson, M., 2012a. A common mechanism of inhibition of the *Mycobacterium tuberculosis* mycolic acid biosynthetic pathway by isoxyl and thiacetazone. *Journal of Biological Chemistry*, 287(46), 38434-38441.

Grzegorzewicz, A.E., Pham, H., Gundi, V.A., Scherman, M.S., North, E.J., Hess, T., Jackson, M., 2012b. Inhibition of mycolic acid transport across the *Mycobacterium tuberculosis* plasma membrane. *Nature Chemical Biology*, 8(4), 334-341.

Gupta, P., Bajpai, S.K., Chandra, K., Singh, K.L., Tandon, J.S., 2005. Antiviral profile of *Nyctanthes arbortristis* L. against encephalitis causing viruses. *Indian Journal of Experimental Biology*, 43, 1156-1160.

Gupta, R., Thakur, B., Singh, P., Singh, H.B., Sharma, V.D., Katoch, V.M., Chauhan, S.V.S., 2010. Anti-tuberculosis activity of selected medicinal plants against multi-drug resistant *Mycobacterium tuberculosis* isolates. *Indian Journal of Medical Research*, 131(6), 809-813.

Harwood, H.J., Greene, Y.J., Stacpoole, P.W., 1986. Inhibition of human leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by ascorbic acid. An effect mediated by the free radical monodehydroascorbate. *Journal of Biological Chemistry*, 261(16), 7127-7135.

Ikonen, E., 2008. Cellular cholesterol trafficking and compartmentalization. *Nature Reviews Molecular Cell Biology*, 9(2), 125-138.

Itoh, M., Hiwatashi, K., Abe, Y., Kimura, F., Toshima, G., Takahashi, J., Hata, K., 2009. Lupeol reduces triglyceride and cholesterol synthesis in human hepatoma cells. *Phytochemistry Letters*, 2(4), 176-178.

Jimenez-Arellanes, A., Luna-Herrera, J., Cornejo-Garrido, J., López-García, S., Castro-Mussot, M.E., Meckes-Fischer, M., Hernández-Pando, R., 2013. Ursolic and oleanolic acids as antimicrobial and immunomodulatory compounds for tuberculosis treatment. *BMC Complementary and Alternative Medicine*, 13(1), 1-11.

Joshi, J.M., 2011. Tuberculosis chemotherapy in the 21st century: Back to the basics. *Lung India: Official Organ of Indian Chest Society*, 28(3), 193-200.

Kang, J., Guo, C., Thome, R., Yang, N., Zhang, Y., Li, X., Cao, X., 2018. Hypoglycemic, hypolipidemic and antioxidant effects of iridoid glycosides extracted from *Corni fructus*: Possible involvement of the PI3K–Akt/PKB signaling pathway. *RSC Advances*, 8(53), 30539-30549.

Kazuhiko, T., Masao, K., Akira, E., 1977. Time-dependent, irreversible inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by the antibiotic citrinin. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 488(1), 97-101.

Khanna, A.K., Chander, R., Kapoor, N.K., Dhawan, B.N., 1994. Hypolipedaemic activity of picroliv in albino rats. *Phytotherapy Research*, 8(7), 403-407.

Khatune, N.A., Mosaddik, M.A., Haque, M.E., 2001. Antibacterial activity and cytotoxicity of *Nyctanthes arbor-tristis* flowers. *Fitoterapia*, 72(4), 412-414.

Kim, H., Mok, J.H., Kang, B., Lee, T., Lee, H.K., Jang, H.J., Jeon, D., 2019. Trend of multidrug and fluoroquinolone resistance in *Mycobacterium tuberculosis* isolates from 2010 to 2014 in Korea: a multicenter study. *The Korean Journal of Internal Medicine*, 34(2), 344-352.

Kirmizibekmez, H., Çalıs, I., Perozzo, R., Brun, R., Dönmez, A.A., Linden, A., Tasdemir, D., 2004. Inhibiting activities of the secondary metabolites of *Phlomis brunneogaleata* against parasitic protozoa and plasmodial enoyl-ACP reductase, a crucial enzyme in fatty acid biosynthesis. *Planta Medica*, 70(8), 711-717.

Korf, J., Stoltz, A., Verschoor, J., De Baetselier, P., Grooten, J., 2005. The *Mycobacterium tuberculosis* cell wall component mycolic acid elicits pathogen-associated host innate immune responses. *European Journal of Immunology*, 35(3), 890-900.

Kul, P.G., Buddhi, B.C., Mamata, B., Laxman, B., 2015. Anti-microbial and anti-diabetic activity of *Nyctanthes arbor-tristis*. *World Journal of Pharmacy and Pharmaceutical Sciences (WJPPS)*, 4(5), 1031-1040.

Kumar, P., Singh, A., Sharma, U., Singh, D., Dobhal, M.P., Singh, S., 2013. Anti-mycobacterial activity of plumericin and isoplumericin against MDR *Mycobacterium tuberculosis*. *Pulmonary Pharmacology & Therapeutics*, 26(3), 332-335.

Lawal, I.O., Grierson, D.S., Afolayan, A.J., 2014. Phytotherapeutic information on plants used for the treatment of tuberculosis in Eastern Cape Province, South Africa. *Evidence-Based Complementary and Alternative Medicine*, 2014, 735423.

Lee, W., VanderVen, B.C., Fahey, R.J., Russell, D.G., 2013. Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. *Journal of Biological Chemistry*, 288(10), 6788-6800.

Li, P., Tian, W., Wang, X., Ma, X., 2014. Inhibitory effect of desoxyrhaponticin and rhaponticin, two natural stilbene glycosides from the Tibetan nutritional food *Rheum tanguticum* Maxim. ex Balf., on fatty acid synthase and human breast cancer cells. *Food & Function*, 5(2), 251-256.

Lobato, L.S., Rosa, P.S., Ferreira, J.D.S., Neumann, A.D.S., da Silva, M.G., do Nascimento, D.C., Lara, F.A., 2014. Statins increase rifampin mycobactericidal effect. *Antimicrobial Agents and Chemotherapy*, 58(10), 5766-5774.

Manjelievskaia, J., Erck, D., Piracha, S., Schrager, L., 2016. Drug-resistant TB: deadly, costly and in need of a vaccine. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 110(3), 186-191.

Mariita, R., Ogol, C.K.P.O., Oguge, N., Okemo, P., 2010. Antitubercular and phytochemical investigation of methanol extracts of medicinal plants used by the Samburu community in Kenya. *Tropical Journal of Pharmaceutical Research*, 9(4), 379-385.

McIlleron, H., Wash, P., Burger, A., Norman, J., Folb, P.I., Smith, P., 2006. Determinants of rifampin, isoniazid, pyrazinamide, and ethambutol pharmacokinetics in a cohort of tuberculosis patients. *Antimicrobial Agents and Chemotherapy*, 50(4), 1170-1177.

Mensink, R.P., 2005. Effects of stearic acid on plasma lipid and lipoproteins in humans. *Lipids*, 40(12), 1201-1205.

Middleton, B., Kok-Pheng, H., 1982. Inhibition of hepatic S-3-hydroxy-3-methylglutaryl-CoA reductase and *in vivo* rates of lipogenesis by a mixture of pure cyclic monoterpenes. *Biochemical Pharmacology*, 31(18), 2897-2901.

Miner, M.D., Chang, J.C., Pandey, A.K., Sassetti, C.M., Sherman, D.R., 2009. Role of cholesterol in *Mycobacterium tuberculosis* infection. *Indian Journal of Experimental Biology*, 47, 407-411.

Mishra, V., Shukla, A., Pandeti, S., Barthwal, M.K., Pandey, H.P., Palit, G., Narender, T., 2013. Arbortristoside-A and 7-*O*-*trans*-cinnamoyl-6β-hydroxyloganin isolated from *Nyctanthes arbortristis* possess anti-ulcerogenic and ulcer-healing properties. *Phytomedicine*, 20(12), 1055-1063.

Miyake, Y., Suzuki, E., Ohya, S., Fukumoto, S., Hiramitsu, M., Sakaida, K., Furuichi, Y., 2006. Lipid-lowering effect of eriocitrin, the main flavonoid in lemon fruit, in rats on a high-fat and high-cholesterol diet. *Journal of Food Science*, 71(9), S633-S637.

Morbidoni, H.R., Vilchèze, C., Kremer, L., Bittman, R., Sacchettini, J.C., Jacobs Jr, W.R., 2006. Dual inhibition of mycobacterial fatty acid biosynthesis and degradation by 2-alkynoic acids. *Chemistry & Biology*, 13(3), 297-307.

Muñoz-Elías, E.J., McKinney, J.D., 2005. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nature Medicine*, 11(6), 638-644.

Murti, K., Kaushik, M., Kaushik, A., 2012. Evaluation of hyypoglycemic and hypolipidemic activity of *Nyctanthes arbortristis* linn against streptozotocin induced diabetic rats. *American Journal of Pharmacology and Toxicology*, 7(1), 8-11.

Ngo, S.C., Zimhony, O., Chung, W.J., Sayahi, H., Jacobs Jr, W.R., Welch, J.T., 2007. Inhibition of isolated *Mycobacterium tuberculosis* fatty acid synthase I by pyrazinamide analogs. *Antimicrobial Agents and Chemotherapy*, 51(7), 2430-2435.

Omar, S.H., 2010. Oleuropein in olive and its pharmacological effects. *Scientia Pharmaceutica*, 78(2), 133-154.

Ouellet, H., Johnston, J.B., de Montellano, P.R.O., 2011. Cholesterol catabolism as a therapeutic target in *Mycobacterium tuberculosis*. *Trends in Microbiology*, 19(11), 530-539.

Pahan, K., 2006. Lipid-lowering drugs. *Cellular and Molecular Life Sciences CMLS*, 63(10), 1165-1178.

Palu, A.K., Brown, A., Deng, S., Norman, K., West, B., 2012. The Effects of Noni (*Morinda citrifolia* L.) Fruit Juice on Cholesterol Levels: A Mechanistic Investigation and an Open Label Pilot Study. *Journal of Applied Pharmaceutical Science*, 2, 25-30.

Pandey, A.K., Sassetti, C.M., 2008. Mycobacterial persistence requires the utilization of host cholesterol. *Proceedings of the National Academy of Sciences*, 105(11), 4376-4380.

Parihar, S.P., Guler, R., Khutlang, R., Lang, D.M., Hurdayal, R., Mhlanga, M.M., Brombacher, F., 2014. Statin therapy reduces the *Mycobacterium tuberculosis* burden in human macrophages and in mice by enhancing autophagy and phagosome maturation. *The Journal of Infectious Diseases*, 209(5), 754-763.

Pattanayak, M., Seth, P.K., Smita, S., Gupta, S.K., 2009. Geraniol and limonene interaction with 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase for their role as cancer chemo-preventive agents. *Journal of Proteomics & Bioinformatics*, 2, 466-474.

Podust, L.M., Yermalitskaya, L.V., Lepesheva, G.I., Podust, V.N., Dalmasso, E.A., Waterman, M.R., 2004. Estriol bound and ligand-free structures of sterol 14α-demethylase. *Structure*, 12(11), 1937-1945.

Rahman, M.M., Rokeya, B., Shahjahan, M., Ahmed, T., Roy, S.K., Ali, L., 2013. Hypoglycemic Effect of *Nyctanthes arbortristis* Linn Extracts in Normal and Streptozotocin-Induced Diabetic Rats. *Malaysian Journal of Pharmaceutical Sciences*, 11(1), 21-31.

Rangika, B.S., Dayananda, P.D., Peiris, D.C., 2015. Hypoglycemic and hypolipidemic activities of aqueous extract of flowers from *Nycantus arbor-tristis* L. in male mice. *BMC Complementary and Alternative Medicine*, 15(1), 1-9.

Rani, C., Chawla, S., Mangal, M., Mangal, A.K., Kajla, S., Dhawan, A.K., 2012. *Nyctanthes arbor-tristis* Linn. (Night Jasmine): A sacred ornamental plant with immense medicinal potentials. *Indian Journal of Traditional Knowledge*, 11, 427-435.

Rathod, N., Raghuveer, I., Chitme, H.R., Ramesh, C., 2009. Prevention of high-fructose diet induced insulin resistance by *Nyctanthes arbortristis* and *Calotropis gigantea* in rats. *Pharmacognosy Magazine*, 5(19), 58-63.

Rathore, A., Rivastava, V., Srivastava, K.C., Tandon, J.S., 1990. Iridoid glucosides from *Nyctanthes arbor-tristis*. *Phytochemistry*, 29(6), 1917-1920.

Reddy Palvai, V., Urooj, A., 2014. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*ex vivo*) by *Morus indica* (Mulberry). *Chinese Journal of Biology*, 2014, 318561.

Roza, J.M., Xian-Liu, Z., Guthrie, N., 2007. Effect of citrus flavonoids and tocotrienols on serum cholesterol levels in hypercholesterolemic subjects. *Alternative Therapies in Health & Medicine*, 13(6), 44-48.

Russell, D.G., 2003. Phagosomes, fatty acids and tuberculosis. *Nature Cell Biology*, 5(9), 776-778.

Sah, A.K., Verma, V.K., 2012. Phytochemicals and pharmacological potential of *Nyctanthes arbor-tristis*: A comprehensive review. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 3(1), 420-427.

Saukkonen, J.J., Cohn, D.L., Jasmer, R.M., Schenker, S., Jereb, J.A., Nolan, C.M., Sterling, T.R., 2006. An official ATS statement: hepatotoxicity of antituberculosis therapy. *American Journal of Respiratory and Critical Care Medicine*, 174(8), 935-952.

Schafer, G., Guler, R., Murray, G., Brombacher, F., Brown, G.D., 2009. The role of scavenger receptor B1 in infection with *Mycobacterium tuberculosis* in a murine model. *PloS One*, 4(12), e8448.

Schroeder, E.K., de Souza, O.N., Santos, D.S., Blanchard, J.S., Basso, L.A., 2002. Drugs that inhibit mycolic acid biosynthesis in *Mycobacterium tuberculosis*. *Current Pharmaceutical Biotechnology*, 3(3), 197-225.

Sheeba, D.G., Gomathi, K.S., Citarasu, D., 2015. Anti-mycobacterial and phytochemical investigation of methanol extracts of few medicinal plants. *Journal of Chemical and Pharmaceutical Sciences*, 8, 480-486.

Shukla, P., Sharma, A., 2021. Effect of some medicinal plants on growth of *Mycobacterium tuberculosis*, multi drug resistant *Mycobacterium tuberculosis* and *Mycobacterium* other than tuberculosis. *Journal of Microbiology, Biotechnology and Food Sciences*, 2021, 199-201.

Sivakumar, A., Jayaraman, G., 2011. Anti-tuberculosis activity of commonly used medicinal plants of south India. *Journal of Medicinal Plants Research*, 5(31), 6881-6884.

Sullivan, T., Amor, Y.B., 2016. Global introduction of new multidrug-resistant tuberculosis drugs—Balancing regulation with urgent patient needs. *Emerging Infectious Diseases*, 22(3), e151228.

Sundaram, R., Shanthi, P., Sachdanandam, P., 2013. Effect of iridoid glucoside on plasma lipid profile, tissue fatty acid changes, inflammatory cytokines, and GLUT4 expression in skeletal muscle of streptozotocin-induced diabetic rats. *Molecular and Cellular Biochemistry*, 380(1), 43-55.

Takayama, K., Wang, C., Besra, G.S., 2005. Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. *Clinical Microbiology Reviews*, 18(1), 81-101.

Tandon, J.S., Srivastava, V., Guru, P.Y., 1991. Iridoids: a new class of leishmanicidal agents from *Nyctanthes arbortristis*. *Journal of Natural Products*, 54(4), 1102-1104.

Tasdemir, D., Güner, N.D., Perozzo, R., Brun, R., Dönmez, A.A., Calıs, I., Rüedi, P., 2005. Anti-protozoal and plasmodial FabI enzyme inhibiting metabolites of *Scrophularia lepidota* roots. *Phytochemistry*, 66(3), 355-362.

Veen, J., 1995. Drug resistant tuberculosis: back to sanatoria, surgery and cod-liver oil?. *European Respiratory Journal*, 8(7), 1073-1075.

Wang, M.Y., Peng, L., Weidenbacher-Hoper, V., Deng, S., Anderson, G., West, B.J., 2012. Noni juice improves serum lipid profiles and other risk markers in cigarette smokers. *The Scientific World Journal*, 2012, 594657.

West, B.J., Jensen, C.J., Palu, A.K., Deng, S., Wasden, J.A., 2014. *Morinda citrifolia* and iridoid based formulations. U.S. Patent No. 8790727. Washington, DC: U.S. Patent and Trademark Office.

WHO (World Health Organization), 2021. Tuberculosis. https://www.who.int/news-room/fact-sheets/detail/tuberculosis; 2021, Accessed 16 December 2021.

Wright, T.C., Cant, J.P., McBride, B.W., 2002. Inhibition of fatty acid synthesis in bovine mammary homogenate by palmitic acid is not a detergent effect. *Journal of Dairy Science*, 85(3), 642-647.

Yamabe, N., Kang, K.S., Matsuo, Y., Tanaka, T., Yokozawa, T., 2007. Identification of antidiabetic effect of iridoid glycosides and low molecular weight polyphenol fractions of *Corni fructus*, a constituent of Hachimi-jio-gan, in streptozotocin-induced diabetic rats. *Biological and Pharmaceutical Bulletin*, 30(7), 1289-1296.

Yamabe, N., Noh, J.S., Park, C.H., Kang, K.S., Shibahara, N., Tanaka, T., Yokozawa, T., 2010. Evaluation of loganin, iridoid glycoside from *Corni fructus*, on hepatic and renal glucolipotoxicity and inflammation in type 2 diabetic db/db mice. *European Journal of Pharmacology*, 648(1-3), 179-187.

Yang, R.M., Liu, F., He, Z.D., Ji, M., Chu, X.X., Kang, Z.Y., Gao, N.N., 2015. Anti-obesity effect of total phenylpropanoid glycosides from *Ligustrum robustum* Blume in fatty diet-fed mice via up-regulating leptin. *Journal of Ethnopharmacology*, 169, 459-465.

Yew, W.W., Lange, C., Leung, C.C., 2010. Treatment of tuberculosis: update 2010. *European Respiratory Journal*, 37, 441-462.

Zhang, S.Y., Zheng, C.G., Yan, X.Y., Tian, W.X., 2008. Low concentration of condensed tannins from catechu significantly inhibits fatty acid synthase and growth of MCF-7 cells. *Biochemical and Biophysical Research Communications*, 371(4), 654-658.

Zheng, Y., Jiang, X., Gao, F., Song, J., Sun, J., Wang, L., Zhang, H., 2014. Identification of plant-derived natural products as potential inhibitors of the *Mycobacterium tuberculosis* proteasome. *BMC Complementary and Alternative Medicine*, 14(1), 400.

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RESEARCH ARTICLE

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Phytomelatonin content in *Valeriana officinalis* L. and some related phytotherapeutic supplements

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ABSTRACT

The use of medicinal plants as an alternative phytotherapeutic remedy against mild illnesses and dysfunctions is increasingly embraced by people. Among these dysfunctions, episodes of nervousness and anxiety due to lack of sleep and insomnia are becoming more and more frequent among the population. To remedy these problems, several plants with sedative activity are recommended. In particular, valerian root (*Valeriana officinalis* L.) is the most recommended and studied with a significant difference. This study presented a quantification of the phytomelatonin contents in valerian root and several related and recommended herb supplements against nervousness, anxiety, and insomnia. The results showed the presence of phytomelatonin in all the samples analyzed. The high phytomelatonin contents in valerian root and its supplements indicated that, in addition to the known constituents of valerian root such as valerenic acid, phytomelatonin also contributed to the phytotherapeutic activity of this plant since the relaxing and sleep-inducing activity of melatonin is well documented. The recommended daily doses of valerian are analyzed according to their phytomelatonin content, and recommendations are given on the possible synergistic action of the components of valerian as a relaxant and sleep inducer in patients with these dysfunctions. It is also recommended to document the phytomelatonin contents in phytotherapeutic preparations.

1. Introduction

A medicinal herb is a plant containing some part/extract that can be used to treat an affectation or disease. The different presentation forms of herb supplements are worldwide products used for the treatment of minor human pathologies or dysfunctions. In the USA, these food supplements are regulated by the American Food and Drug Administration (FDA), which uses the term dietary supplements for products that have not been subject to control or evaluation and stipulate that these dietary supplements must be labeled with the annotation that they have not been evaluated by the FDA. In the European Union (EU), more restrictive legislation is applied by the European Medicines Agency (EMA) (EMA-HMP, 2018; Vanaclocha and Cañigueral Folcara, 2019).

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Generally, medicinal herbs and nutraceuticals of plant origin tend to be more accepted by consumers than others. In this sense, nutrition-based healthcare during the history of humankind can be explained by the funny commentary of Rowe (1999):

2000 B.C. - Here, eats this root.
1000 A.D. - That root is heathen. Here, says this prayer.
1850 A.D. - That prayer is superstition. Here, drinks this potion.
1940 A.D. - That potion is snake oil. Here, swallow this pill.
1985 A.D. - That pill is ineffective. Here, takes this antibiotic.
2000 A.D. - That antibiotic is artificial. Here, eats this root.

The trend of using pharmaceutical preparations of natural origin is increasing owing to the better knowledge of its efficacy and safety. Due to increased stress and sleep difficulties leading to episodes of anxiety, many people consider the consumption of medicinal plants as a possible alternative solution. Some medicinal plants have the property of being sedatives acting on the central nervous system, decreasing nervous excitement, being used as a treatment against anxiety, insomnia, and nervousness (Hadley and Petry, 2003; Wheatley, 2005; Singh and Sharma, 2020).

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Valeriana officinalis L. (valerian) is one of the plants on which more clinical studies have been published, demonstrating its effectiveness as a mild tranquilizer in cases of generalized nervousness, insomnia, restlessness, and moderate anxiety states. Its non-toxicity has been demonstrated in therapeutic doses. Valerian root is used as a sedative or sleep aid. It is worldwide available as a food supplement. Valerian supplements, with *V. officinalis* roots as the main component, were used to improve sleep quality and minimize anxiety, stress, or moderate nervousness, all thanks to its sedative and anxiolytic properties. Among the recommendations for valerian root, approved by the European Scientific Cooperative on Phytotherapy (ESCOP), are the relief of mild nervous tension episodes and difficulty falling asleep. In addition, EMA approves its traditional use to relieve mild mental stress symptoms and fall asleep (HMPC-EMA, 2016; Hadley and Petry, 2003).

Multiple studies of valerian roots and their possible effects on the improvement and induction of sleep have been conducted in recent decades. Generally, randomized and double-blind clinical trials have shown that extracts of valerian root and valerian supplements allow a higher quality in sleep parameters, facilitating relaxation, reducing the time needed to fall asleep, and deeper sleep phases than placebo. Nevertheless, some difficulties in interpreting the results appear because of the different dosage, origin, and purity of the herbaceous extracts used. However, the effects are not immediate, and treatment of 2-4 weeks is required to achieve a significant improvement, without risk of dependence or adverse effects, which differentiates the action of valerian roots from that of synthetic hypnotics and benzodiazepines (Tammadon et al., 2021; Shinjyo et al., 2020; Palmieri et al., 2017; Taavoni et al., 2011; Barton et al., 2011; Dimpfel and Suter, 2008; Miyasaka et al., 2006; Bent et al., 2006).

Furthermore, melatonin supplements are also used to improve sleep quality and sleep disorders, including insomnia, and avoid jet lag. Also, to prevent or treat states of anxiety or depression (Dahlitz et al., 1991; Ferracioli-Oda et al., 2013; Herxheimer, 2005; Jan et al., 2000; Xie et al., 2017). Melatonin supplements are made with synthetic melatonin, which is cheap and easy to synthesize chemically (He et al., 2003; Thomson et al., 2003; Bartolucci et al., 2016). These supplements can be found in the market in a wide range of form presentations such as tablets, pills, liquid, sublingual, etc. The regulations for these melatonin supplements are different depending on the country, being more strongly regulated in the EU and more laxly by the FDA in the USA (Coppens et al., 2006; Finley et al., 2014). In recent years, it has been proposed that obtaining phytomelatonin (discovered its presence in 1995) from plants (Pérez-Llamas et al., 2020; Arnao and Hernández-Ruiz, 2018) could be a natural alternative in the making of melatonin supplements since, in some cases, by-products of certain toxicity have been detected in synthetic melatonin preparations (Mayeno and Gleich, 1994; Naylor et al., 1999; Williamson et al., 1998). Phytomelatonin is present in all the plants studied, generally at low concentrations, being its most relevant content in aromatic and medicinal plants (Arnao, 2014; Arnao and Hernández-Ruiz, 2015; 2018; Cheng et al., 2021).

In this paper, a relationship between the sedative capacity of valerian roots and supplements and phytomelatonin content was studied. Valerian dry roots and herb supplements with/without valerian root were analyzed to know its phytomelatonin content and establish a possible synergic effect of traditional valerian components and the novel phytomelatonin determination in plants. A study of the recommended maximum daily dose of herb supplements and the phytomelatonin content was presented.

2. Materials and methods

2.1. Chemicals

The chemicals, solvents (methanol, acetonitrile, ethyl acetate), and reagents used were from Sigma-Aldrich Co. (Madrid, Spain). Milli-Q system (Milli-Q Corp, Merck, Darmstadt, Germany) ultra-pure water was used.

2.2. Plant material

Dried roots of *V. officinalis* were obtained from Murciana de Herboristeria S.A. (Cobatillas, Murcia, Spain), a specialized company in medicinal and aromatic herbs. Table 1 shows the technical sheet of valerian root used.

Table 1. Physico-chemical and microbiological characterization of *V. officinalis* samples

Product	Valerian roots
Denomination	The healthy, clean, and dried root of *V. officinalis*
Origin	Poland
Organoleptic characteristics	
Aspect:	Correct
Smell:	Characteristic, without the presence of strange odors
Color:	Brown
Physico-chemical characteristics	
Humidity (%)	12,00 Máx.
Total ash (%)	12,00 Máx.
Insoluble ash (%)	5,00 Máx.
Microbiological characteristics	
Escherichia coli (ufc/g)	≤100
Salmonella spp.	Absence / 25 gr
Total aerobics (ufc/g)	≤10.000.000
Molds and yeasts (ufc/g)	≤100.000

Moreover, three herbal supplements with sedative properties obtained from a local pharmacy office were used in the study. These herb supplements are commercialized in Spain and many other products with similar compositions in the EU. The supplements were: Valeriana Eladiet from Eladiet Lab. (Barcelona, Spain), Valeriana Deliplus from Korott Lab (Alicante, Spain), and Neuro Balance from Salus Lab (Bruckmühl, Germany), all recommended as sedatives and as a sleep aid. Table 2 shows the ingredients and composition of these herb supplements.

Table 2. Ingredients and composition of the herb supplements studied

Product	**Ingredients**	**Deck ingredients**
Valeriana Eladiet	60 pills (330 mg)	
	Valerian officinalis root (257 mg)	Hydroxypropylmethylcellulose
	Silicon dioxide	Microcrystalline cellulose
	Hydroxypropylcellulose	Stearic acid
	Magnesium stearate	Erythrosine
	Polyethylene glycol 6000	Indigo carmine
		Titanium dioxide
Valeriana Deliplus	60 pills (520 mg)	
	Valerian officinalis root (400 mg)	Gelatin
	Magnesium stearate	
Neuro Balance	10 ml	
	Aqueous extract of:	Juice concentrates of:
	Lemon balm leaves	Mango puree
	Lavender flowers	Guava puree
	Mint leaves	Ashwagandha root extract
	Passiflora grass	Carob flour
	Rosemary leaves	
	Vitamin C, Vitamin B12	
	Natural flavorings	

2.3. Extraction of phytomelatonin

Samples for phytomelatonin analysis were prepared according to Hernández-Ruiz and Arnao (2008) and Arnao and Hernández-Ruiz (2009). Briefly, samples of valerian roots and herb supplements (0.3 g DW) were placed in vials containing ethyl acetate (5 ml). After leaving overnight (15 h) at 4 °C in darkness with shaking, the tissue was discarded after being washed with the respective solvent (0.5 ml). The extract and washing solution from each sample was evaporated to dryness under vacuum using a SpeedVac (ThermoSavant SPD111V, Thermo-Fisher Sci, Waltham, MA, USA) coupled to a refrigerated RVT400 vapor trap. The residue was redissolved in acetonitrile (1 ml), filtered (0.2 µm), and analyzed using liquid chromatography with fluorescence detection (LC-FLUO) and by LC with time-of-flight mass spectroscopy (LC-QTOF/MS). The procedures were carried out in artificial dim light.

2.4. Phytomelatonin analysis

Phytomelatonin content in valerian roots and herb supplements was determined by liquid chromatography with fluorescence detection (LC-FLUO) and by LC with time-of-flight mass spectroscopy (LC-QTOF/MS), according to our previous studies (Arnao and Hernández-Ruiz, 2009). A Jasco liquid chromatograph Serie-2000 (Tokyo, Japan) equipped with an online degasser, binary pump, auto multi sampler, thermo-stated column, and a Jasco FP-2020-Plus fluorescence detector were used to measure phytomelatonin levels. An excitation wavelength of 280 nm and an emission wavelength of 350 nm were selected. A Waters XBridge-C18 (5 µm of particle size), 100 x 2.1 mm LC column (Sigma-Aldrich, Barcelona, Spain), and thermo-stated at 40 °C were used. Solvents A (MilliQ water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) in isocratic mode, at a flow rate of 0.2 ml/min, were used for the compound separation. The data were analyzed using the Jasco ChromNAV v.1.09.03 Data System Software (Tokyo, Japan). For correct identification, an in-line fluorescence spectral analysis using the Jasco Spectra Manager Software (Tokyo, Japan) compared the excitation and emission spectra of standard melatonin with the corresponding peak of phytomelatonin in the samples. Identification of phytomelatonin in plant extracts was also confirmed using an LC/QTOF-MS system consisting of an Agilent 1290 Infinity II Series LC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multi sampler module and a high-speed binary pump and connected to an Agilent 6550 Q-TOF Mass Spectrometer using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Experimental parameters for LC and Q-TOF were set in MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00). The set parameters and signal corresponding to phytomelatonin were extracted and quantified with an m/z of 233.1285, according to (Hernández-Ruiz and Arnao, 2008).

2.5. Statistical analysis

For collecting all data, differences were determined using the SPSS 10 program (SPSS Inc., Chicago, USA), applying the LSD multiple range tests to establish significant differences at $p < 0.05$. The results are expressed as mean ± standard error (SE, $n = 5$).

3. Results and discussion

A peak corresponding to phytomelatonin was detected by LC-FLUO in the described conditions at ~ 12.3 min, as can be seen in Figure 1A, where a chromatogram of standard melatonin appears. Also, a calibration curve of standard melatonin in the range used is shown (Figure 1B). In the same conditions, the presence of phytomelatonin in a valerian root sample was indicated by its peak retention time (Figure 2) and from the respective excitation and emission spectra, including a comparison of the first- and second-order derivatives from each spectrum (Arnao and Hernández-Ruiz, 2009). The presence of phytomelatonin in the samples was confirmed by spiking the standard (at ng levels) into the root sample extracts and observing the increase in the putative melatonin peak. Also, confirmation was obtained by LC/QTOF-MS analysis (Hernández-Ruiz and Arnao, 2008).

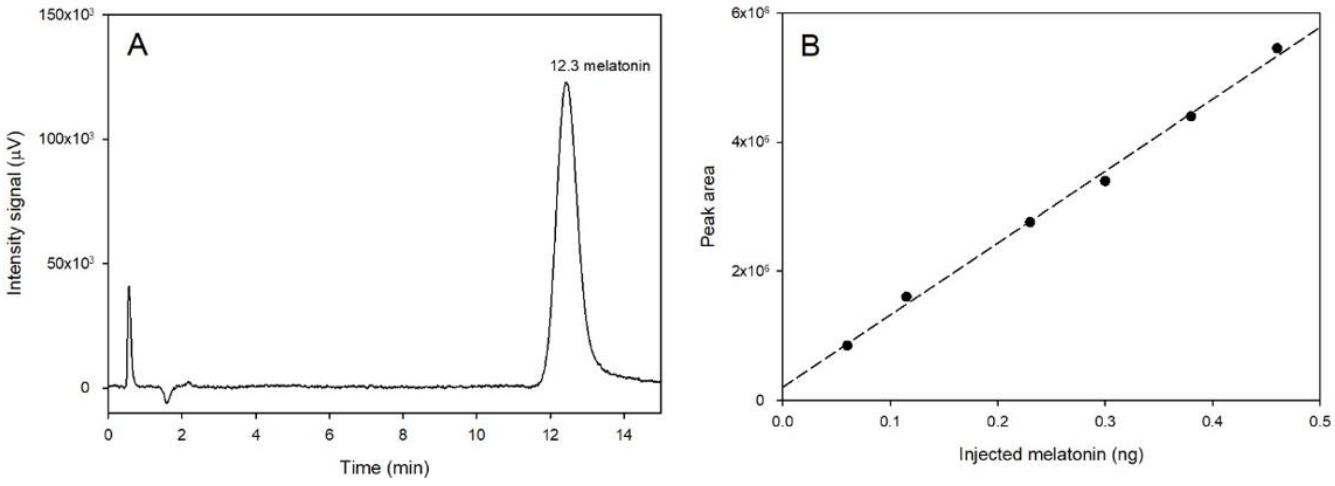

Figure 1. Panel A: Representative chromatogram of standard melatonin (116 pg) measured by LC with fluorescence detection with λexc = 280 nm and λemi = 350 nm. The melatonin peak at the retention time of 12.3 min is marked. Panel B: Linear regression curve of standard melatonin. Circles represent the means of three replicates; SE is included in the size of the symbols.

Table 3 shows the phytomelatonin quantitation in each studied sample. All the samples contained significant amounts of phytomelatonin. Phytomelatonin levels in edible plants range from nanograms to micrograms per gram of plant tissue. In general, seeds, leaves, stems, seedlings, and roots show the highest phytomelatonin levels and fruits the lowest. Medicinal/aromatic plants have significantly higher levels than seeds and fruits. Additionally, growing conditions and harvesting, among other

factors, affect phytomelatonin levels in plant tissues (Arnao, 2014; Arnao and Hernández-Ruiz, 2013; 2014; 2015).

Valerian root samples showed a high phytomelatonin content. Herb supplements containing valerian also presented similar contents. The differences between them (from 0.96 to 2.09 in Table 3) can be explained due to the variability of valerian root sources, a common aspect in herb supplements. When the phytomelatonin content is calculated by pill, considering the valerian dosage in each herb supplement (see technical sheets in material and methods), Valeriana Deliplus showed the highest dose (0.8 µg/pill).

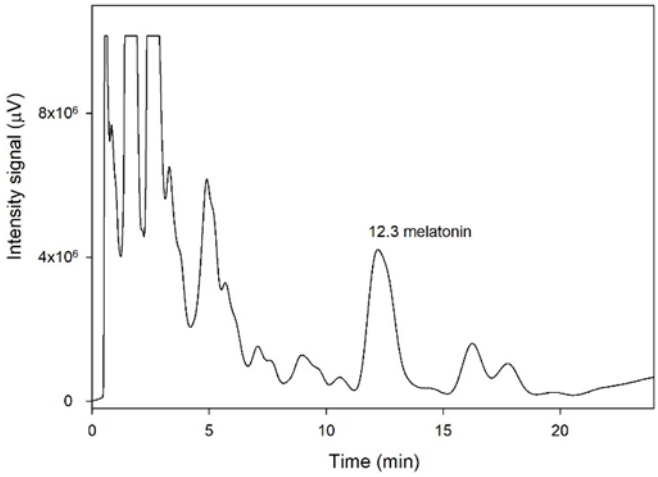

Figure 2. Representative chromatogram of valerian root samples measured by LC with fluorescence detection with λexc = 280 nm and λemi = 350 nm. The melatonin peak at the retention time of 12.3 min is marked.

According to a meta-analysis study on thirteen valerian products commonly used in the USA, which were evaluated by a reference laboratory, the recommended doses ranged from 75 to 3000 mg per day (Bent et al., 2006). In the EU, EMA recommends a daily dose of 400-600 mg of extract to relieve nervous tension, 1-3 times a day. Therefore, an MRDD is 1200-1800 mg of valerian extract (HMPC-EMA, 2016). However, ESCOP recommends up to 1-3 g of the dry herb of MRDD in infusion. Considering these approximate data, with ESCOP recommendation (MRDD of 3 g), the intake of phytomelatonin from valerian root infusion can become 4.68 µg/day. In the case of herb supplements, considering the recommendation by each lab, phytomelatonin intake in Valeriana Deliplus was higher than Valeriana Eladiet and higher than in valerian roots (Table 3).

Table 3. Content of phytomelatonin in the four studied samples

Samples	Phytomelatonin content		
	µg/g DW	µg/pill	µg/MRDD**
V. officinalis (root)	1.56 ± 0.068*	-	4.68
Valeriana Eladiet	0.96 ± 0.051	0.25	0.75
Valeriana Deliplus	2.09 ± 0.087	0.8	6.27
Neuro Balance	0.36 ± 0.008	3.6	7.2

*Average data ± SE (*n* = 5); **Maximum Recommended Daily Dose

According to the European Pharmacopoeia, the main constituents of valerian root are essential oils (0.3-1.1 %) and amino acids such as gamma-aminobutyric acid (GABA), glutamine, and arginine, in relatively high proportions. It also contains flavonoids (hesperidin and 6-methyl-apigenin), lignans (8'-hydroxypinosinol), traces of alkaloids (valerianine, methylpyrrolyl ketone), and several acidic compounds such as valerenic, formic, acetic, etc. Regarding pharmacological activity, valerian root preparations have a sedative and sleep-inducing action through a mechanism that would increase GABA transmission by increasing the concentration of GABA in the synaptic space due to an inhibition of its catabolism and reuptake. The cause of the inhibition of GABA catabolism could be valerenic acid, a sesquiterpenoid present in valerian essential oil (Figure 3). Most of the commercialized extracts were standardized to 0.8 % valerenic acid (Boonstra et al., 2015; Trauner et al., 2008; Vanaclocha and Cañigueral Folcara, 2019).

As is known, melatonin is a hormone that the brain produces in response to darkness to help the body fall asleep. It is also applied to prevent sleep disturbances from jet lag to improve insomnia and sleep efficacy/quality. Curiously, in valerian products (dry roots and supplements) the phytomelatonin contents never appear described. This is because phytochemical laboratories do not have the analysis of melatonin of plant origin among their routines since it is a relatively new molecule in the field of phytotherapy. Certainly, the phytomelatonin contained in valerian preparations could play an important role in the sedative and sleep-inducing action, hitherto attributed exclusively to valerenic acid. Although structurally, phytomelatonin and valerenic acid are not very similar (Figure 3), it is possible that both molecules contribute to the phytotherapeutic activity, and even with possible synergistic effects.

As an example of the importance of phytomelatonin in herb supplements, Table 3 also shows the phytomelatonin content of Neuro Balance samples. As it can be seen in the technical sheet (see material and methods), it is a natural multicomponent supplement based on juices and herbs, highlighting among its components the presence of Passiflora (*Passiflora incarnata* L.) and Ashwagandha (*Withania somnifera* L.) herbs. Ashwagandha root, also known as Indian ginseng or sleep herb, is used to induce and improve the quality of sleep in people suffering from insomnia and stress. Passiflora is also recommended in the case of insomnia and to help sleep-in. The analysis shows that Neuro Balance contains a low level of phytomelatonin (0.36 µg/ml), but considering the recommended daily dose (20 ml), a person can intake up to 7.2 µg of phytomelatonin a day, possibly enough to induce sleep-in. The exact origin of the phytomelatonin in this supplement is difficult to know, possibly from Passiflora and Ashwagandha but also from the other plant components of the mixture. In any case, this would be a good

example that herb supplements "designed to aid sleep" tend to contain phytomelatonin and that consumers should be made aware of this fact.

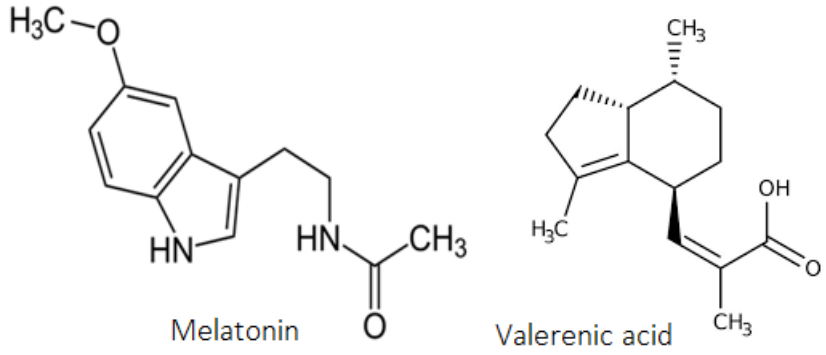

Figure 3. Chemical structures of melatonin and valerenic acid

Currently, studies with valerian focused on treating anxiety and insomnia continue to advance with very positive results (Das et al., 2021; Borrás et al., 2021). Also, in trials in patients with neuropathies (Soltani et al., 2021), mental disorders such as Alzheimer's (Marde et al., 2022), and in pregnancy (Kennedy et al., 2013), the beneficial effects of valerian extracts, as well as other phytotherapeutics, have been proven with interesting results. Another attractive line of research with valerian is the studies against skeletal muscle atrophy, of great interest in athletes (Kim et al., 2022). Also, in *in vitro* studies, no harmful effects have been detected at high doses of tranquilizing herbs, including valerian (Spiess et al., 2021). However, some recent studies suggest that these herbs can alter the pharmacokinetic response to drugs (Matura et al., 2021).

4. Conclusions

Our recommendation, considering the results, is that the phytomelatonin content in valerian supplements and other sleep-in inductors/improvers be determined and reported in the prospectuses or technical sheets of the product so that the consumer is aware that other compounds contained in the preparations can help you in therapeutic treatment. Also, research on valerian preparations should be promoted to understand the possible synergistic interaction of valerenic acid and phytomelatonin to establish and quantify the role of each of them. Finally, the design of herb supplements to improve sleep with plant components rich in phytomelatonin may be a future strategy of great interest. Currently, new research on the possible role of phytomelatonin in other herb supplements related to anxiety and sleep quality is in perspective.

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None.

Conflict of interest

The author confirms that there are no known conflicts of interest.

CRediT authorship contribution statement

Marta Losada: Formal analysis, Writing original draft, Writing-reviewing & editing
Antonio Cano: Methodology, Writing-reviewing & editing
Josefa Hernandez-Ruiz: Data curation, Supervision, Writing-reviewing & editing
Marino B. Arnao: Conceptualization, Formal analysis, Methodology, Writing original draft, Supervision, Writing-reviewing & editing

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Supplementary File

None.

References

Arnao, M.B., 2014. Phytomelatonin: discovery, content, and role in plants. *Advances in Botany*, e815769.
Arnao, M.B., Hernández-Ruiz, J., 2009. Assessment of different sample processing procedures applied to the determination of melatonin in plants. *Phytochemical Analysis*, 20(1), 14-18.
Arnao, M.B., Hernández-Ruiz, J., 2013. Growth conditions influence the melatonin content of tomato plants. *Food Chemistry*, 138(2-3), 1212-1214.
Arnao, M.B., Hernández-Ruiz, J., 2014. Melatonin: plant growth regulator and/or biostimulator during stress?. *Trends in Plant Science*, 19(12), 789-797.
Arnao, M.B., Hernández-Ruiz, J., 2015. Phytomelatonin: Searching for plants with high levels as a natural source of nutraceuticals. In F. R. S. Atta-Ür-Rahman (Ed.), Studies in Natural Products Chemistry (Bioactive Natural Products) vol. 46 (Vol. 1–11, pp. 519–545). Elsevier Science Publishers.
Arnao, M.B., Hernández-Ruiz, J., 2018. The potential of phytomelatonin as a nutraceutical. *Molecules*, 23(1), 238.
Bartolucci, S., Mari, M., Di Gregorio, G., Piersanti, G., 2016. Observations concerning the synthesis of tryptamine homologues and branched tryptamine derivatives via the borrowing hydrogen process: synthesis of psilocin, bufotenin, and serotonin. *Tetrahedron*, 72(18), 2233-2238.
Barton, D.L., Atherton, P.J., Bauer, B.A., Moore Jr, D.F., Mattar, B.I., LaVasseur, B.I., Loprinzi, C.L., 2011. The use of *Valeriana officinalis* (valerian) in improving sleep in patients who are undergoing treatment for cancer: A phase III randomized, placebo-controlled, double-blind study: NCCTG Trial, N01C5. *The Journal of Supportive Oncology*, 9(1), 24-31.
Bent, S., Padula, A., Moore, D., Patterson, M., Mehling, W., 2006. Valerian for sleep: a systematic review and meta-analysis. *The American Journal of Medicine*, 119(12), 1005-1012.
Boonstra, E., De Kleijn, R., Colzato, L.S., Alkemade, A., Forstmann, B.U., Nieuwenhuis, S., 2015. Neurotransmitters as food supplements: the effects of GABA on brain and behavior. *Frontiers in Psychology*, 6, 1520.
Borrás, S., Martínez-Solís, I., Ríos, J.L., 2021. Medicinal Plants for Insomnia Related to Anxiety: An Updated Review. *Planta Medica*, 87, 738–753.
Cheng, G., Ma, T., Deng, Z., Gutiérrez-Gamboa, G., Ge, Q., Xu, P., Sun, X., 2021. Plant-derived melatonin from food: A gift of nature. *Food & Function*, 12(7), 2829-2849.

Coppens, P., Da Silva, M.F., Pettman, S., 2006. European regulations on nutraceuticals, dietary supplements and functional foods: a framework based on safety. *Toxicology*, 221(1), 59-74.
Dahlitz, M., Alvarez, B., Parkes, J.D., English, J., Arendt, J., Vignau, J., 1991. Delayed sleep phase syndrome response to melatonin. *The Lancet*, 337(8750), 1121-1124.
Das, G., Shin, H.S., Tundis, R., Gonçalves, S., Tantengco, O.A.G., Campos, M.G., Patra, J.K., 2021. Plant Species of Sub-Family Valerianaceae—A Review on Its Effect on the Central Nervous System. *Plants*, 10(5), 846.
Dimpfel, W., Suter, A., 2008. Sleep improving effects of a single dose administration of a valerian/hops fluid extract—A double blind, randomized, placebo-controlled sleep-EEG study in a parallel design using electrohypnograms. *European Journal of Medical Research*, 13(5), 200–204.
EMA-HMP, 2018, September 17. Herbal medicinal products . European Medicines Agency. https://www.ema.europa.eu/en/human-regulatory/herbal-medicinal-products
Ferracioli-Oda, E., Qawasmi, A., Bloch, M.H., 2013. Meta-analysis: melatonin for the treatment of primary sleep disorders. *PloS One*, 8(5), e63773.
Finley, J.W., Finley, J.W., Ellwood, K., Hoadley, J., 2014. Launching a new food product or dietary supplement in the United States: industrial, regulatory, and nutritional considerations. *Annual Review of Nutrition*, 34, 421-447.
Hadley, S.K., Petry, J.J., 2003. Valerian. *American Family Physician*, 67(8), 1755-1758.
He, L., Li, J.L., Zhang, J.J., Su, P., Zheng, S.L., 2003. Microwave assisted synthesis of melatonin. *Synthetic Communications*, 33(5), 741-747.
Hernández-Ruiz, J., Arnao, M.B., 2008. Distribution of melatonin in different zones of lupin and barley plants at different ages in the presence and absence of light. *Journal of Agricultural and Food Chemistry*, 56(22), 10567-10573.
Herxheimer, A., 2005. Jet lag. *Clinical Evidence*, (13), 2178-2183.
HMPC-EMA, 2016. European Union herbal monograph on *Valeriana officinalis* L., radix. Herbal Medicinal Products Committee, EMA/HMPC/150848/2015, 9.
Jan, J.E., Hamilton, D., Seward, N., Fast, D.K., Freeman, R.D., Laudon, M., 2000. Clinical trials of controlled-release melatonin in children with sleep–wake cycle disorders. *Journal of Pineal Research*, 29(1), 34-39.
Kennedy, D.A., Lupattelli, A., Koren, G., Nordeng, H., 2013. Herbal medicine use in pregnancy: results of a multinational study. BMC Complementary and Alternative Medicine, 13(1), 355.
Kim, Y.I., Lee, H., Nirmala, F.S., Seo, H.D., Ha, T.Y., Jung, C.H., Ahn, J., 2022. Antioxidant Activity of *Valeriana fauriei* Protects against Dexamethasone-Induced Muscle Atrophy. *Oxidative Medicine and Cellular Longevity*, 2022, 3645431.
Marde, V.S., Atkare, U.A., Gawali, S.V., Tiwari, P.L., Badole, S.P., Wankhede, N.L., Kale, M.B., 2022. Alzheimer's disease and sleep disorders: Insights into the possible disease connections and the potential therapeutic targets. *Asian Journal of Psychiatry*, 68, 102961.
Matura, J.M., Shea, L.A., Bankes, V.A., 2021. Dietary supplements, cytochrome metabolism, and pharmacogenetic considerations. *Irish Journal of Medical Science*, 1971, 1-9.
Mayeno, A.N., Gleich, G.J., 1994, July. The eosinophilia-myalgia syndrome: lessons from Germany. *In Mayo Clinic Proceedings* (Vol. 69, No. 7, pp. 702-704). Elsevier.
Miyasaka, L.S., Atallah, Á.N., Soares, B., 2006. Valerian for anxiety disorders. *Cochrane Database of Systematic Reviews*, (4), CD004515.
Naylor, S., Johnson, K.L., Williamson, B.L., Klarskov, K., Gleich, G.J., 1999. Structural characterization of contaminants in commercial preparations of melatonin by on-line HPLC-electrospray ionization-tandem mass spectrometry. *Advances in Experimental Medicine and Biology*, 467, 769–777.
Palmieri, G., Contaldi, P., Fogliame, G., 2017. Evaluation of effectiveness and safety of a herbal compound in primary insomnia symptoms and sleep disturbances not related to medical or psychiatric causes. *Nature and Science of Sleep*, 9, 163-169.
Pérez-Llamas, F., Hernández-Ruiz, J., Cuesta, A., Zamora, S., Arnao, M.B., 2020. Development of a phytomelatonin-rich extract from cultured plants with excellent biochemical and functional properties as an alternative to synthetic melatonin. *Antioxidants*, 9(2), 158.
Rowe, S.B., 1999. What is a Nutrient? Defining the Food-Drug Continuum. In Round table forum, moderator's comments. Georgetown University.
Shinjyo, N., Waddell, G., Green, J., 2020. Valerian root in treating sleep problems and associated disorders-A systematic review and meta-analysis. *Journal of Evidence-Based Integrative Medicine*, 25, 2515690X20967323.
Singh, B., Sharma, R.A., 2020. Secondary Metabolites of Medicinal Plants, 4 Volume Set: Ethnopharmacological Properties, Biological Activity and Production Strategies (1st ed.). Wiley-VCH.
Soltani, A., Bahrami, F., Bahari, Z., Shankayi, Z., Graily-Afra, M., Sahraei, H., 2021. The effects of Valerian on sleep spindles in a model of neuropathic pain. *Sleep Science*, 14(Spec 2), 133-139.
Spiess, D., Winker, M., Chauveau, A., Abegg, V.F., Potterat, O., Hamburger, M., Simões-Wüst, A.P., 2021. Medicinal Plants for the Treatment of Mental Diseases in Pregnancy: An *In Vitro* Safety Assessment. *Planta Medica*.
Taavoni, S., Ekbatani, N., Kashaniyan, M., Haghani, H., 2011. Effect of valerian on sleep quality in postmenopausal women: a randomized placebo-controlled clinical trial. *Menopause*, 18(9), 951-955.
Tammadon, M.R., Nobahar, M., Hydarinia-Naieni, Z., Ebrahimian, A., Ghorbani, R., Vafaei, A.A., 2021. The effects of valerian on sleep quality, depression, and state anxiety in hemodialysis patients: a randomized, double-blind, crossover clinical trial. *Oman Medical Journal*, 36(2), e255.
Thomson, D.W., Commeureuc, A.G., Berlin, S., Murphy, J.A., 2003. Efficient route to the pineal hormone melatonin by radical-based indole synthesis. *Synthetic Communications*, 33(20), 3631-3641.
Trauner, G., Khom, S., Baburin, I., Benedek, B., Hering, S., Kopp, B., 2008. Modulation of GABAA receptors by valerian extracts is related to the content of valerenic acid. *Planta Medica*, 74(1), 19-24.
Vanaclocha, B., Cañigueral Folcara, S., 2019. Fitoterapia. Vademécum de prescripción (5th ed.). Elsevier Science Publishers. https://tienda.elsevier.es/fitoterapia-vademecum-de-prescripcion-9788491132998.html
Wheatley, D., 2005. Medicinal plants for insomnia: a review of their pharmacology, efficacy and tolerability. *Journal of Psychopharmacology*, 19(4), 414-421.
Williamson, B.L., Tomlinson, A.J., Mishra, P.K., Gleich, G.J., Naylor, S., 1998. Structural characterization of contaminants found in commercial preparations of melatonin: similarities to case-related compounds from L-tryptophan associated with eosinophilia-myalgia syndrome. *Chemical Research in Toxicology*, 11(3), 234-240.
Xie, Z., Chen, F., Li, W.A., Geng, X., Li, C., Meng, X., Yu, F., 2017. A review of sleep disorders and melatonin. *Neurological Research*, 39(6), 559-565.

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RESEARCH ARTICLE

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Synthesis, characterization, antibacterial, antioxidant activity, and lipoxygenase enzyme inhibition profile of silver nanoparticles (AgNPs) by green synthesis from *Seseli resinosum* Freyn & Sint

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ABSTRACT

In the study, silver nanoparticles (AgNPs) were successfully synthesized by an environmentally friendly synthesis method using *Seseli resinosum* Freyn & Sint extract. The synthesized silver nanoparticles were characterized by ultraviolet/visible light absorption spectrophotometer (UV-Vis), X-ray diffraction (XRD), and scanning electron microscopy (SEM) analysis. As a result of the characterization, it was determined that 33 nm spherical nanoparticles were formed, showing a spectrum at ~420 nm wavelength. Silver nanoparticles showed a bacteriocidal effect against all bacterial strains. DPPH and ABTS methods were used to examine the antioxidant activities of plant extracts and AgNPs. In DPPH removal activity, AgNPs obtained by green synthesis provided a high rate of inhibition removal compared to the extract. According to this percentage, while silver nanoparticles provided 22% removal, the extract provided 15% removal. In ABTS removal activity, when AgNPs were obtained by green synthesis compared to the extract, silver nanoparticles provided 25% removal, while the extract provided 18% removal. The characterization of silver nanoparticles synthesized by the green synthesis method and their antioxidant activity were investigated, and the obtained values indicate the presence of an antioxidant capacity. In addition, the inhibitory effects of the extract and AgNP on lipoxygenase activity, which has an important place in health, were investigated. It was determined that the aqueous extract of *S. resinosum* and the AgNP synthesized from the extract had lipoxygenase enzyme inhibitory activity.

1. Introduction

Nanoparticles; are materials with sizes ranging from 1-100 nm (Ahmed et al., 2016). Nanoparticles are used in many fields such as bio-medical, catalysis, food, clothing, cosmetics, and electronics (Gopinath et al., 2016; Saravanakumar et al., 2018; Selvakumar et al., 2018). Silver (Ag) has been used in antibacterial applications in many fields for many years due to its activity against bacteria and other microorganisms (Yang et al., 2012); silver nanoparticles (AgNPs) have been reported by some researchers to have strong antibacterial activity (Xu et al., 2011; Kung et al., 2018). Various methods are used to obtain silver nanoparticles. Among these methods, the biological method is preferred more than other (physical and chemical) methods because it is environmentally friendly and economical (Chen et al., 2003; Francis et al., 2017). In the synthesis of silver nanoparticles, the use of plant sources is more economical, and the application processes are easier, so it has attracted more attention recently (Pallela et al., 2018). Free radicals are atoms, molecules, or ions with unpaired electrons that are unstable to react chemically with other molecules. Oxygen-centered free radicals are known as reactive oxygen species (ROS) (Han et al., 2018). Oxidative stress is the result of an imbalance between ROS and antioxidant defenses. Oxidative stress disrupts a number of cellular functions and causes various negative effects as it suppresses the organism's antioxidative defense mechanism. Antioxidants are molecules that can prevent or delay the oxidation of an oxidizable substrate (Halliwell, 1990). Therefore, research on natural antioxidant compounds that can be obtained from plants and can efficiently scavenge free radicals has been increasing in recent years (Sindhi et al., 2013). Lipoxygenases (LOX) enzymes are a group of enzymes

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detected in animals, food yeast, plants, algae, fungi, cyanobacteria, and algae. In the pathway of arachidonic acid metabolism carried out by LOX, substances called reactive oxygen species (ROS) are formed. Other arachidonic acid metabolites and ROS substances formed in this way can cause tumor formation or inflammation (Juntachote and Berghofer, 2005). In addition, LOXs and their metabolites are implicated in many types of human cancers, such as prostate, lung, breast, colon, and other cancer cell lines (Samuelsson et al., 1987). It is very important to find new LOX inhibitors to treat human diseases such as cancer, cardiovascular and neurodegenerative disorders (Kelavkar et al., 2000). LOX is a very important enzyme in the food sector as well as in the health sector, and its characterization and inhibition should be investigated. However, the demand for natural antioxidants has increased recently due to the toxicity of these synthetic compounds (Guzel et al., 2017). This study it is aimed to determine the characterization, antibacterial, antioxidant activity, and lipoxygenase enzyme inhibition profile of AgNPs obtained by using the extracts of *Seseli resinosum* Freyn & Sint by green synthesis method without using toxic and expensive chemicals. Characterization was done by SEM, UV-Vis, and XRD analysis. The effects of AgNPs on antibacterial, antioxidant, and lipoxygenase enzyme inhibition were investigated. In conclusion, extracts and AgNPs may be promising antioxidants for the health industry.

2. Materials and methods

2.1. Preparation of plant sample and extract

After the plant sample used in the study was collected, it was thoroughly washed in tap water, dried in a cool and moisture-free environment, and made ready for grinding (Davis, 1970). Distilled water was used as a solvent to prepare the plant extract, which was dried at room temperature. The leaf part of the plant was ground with the help of liquid nitrogen by crushing in a mortar. 10 g of the ground plant sample was weighed, and 150 ml of distilled water was used as a solvent. The magnetic heater was also set at 80 °C and extracted for 4 hours. At the end of the period, filtration was performed using filter papers. The extract obtained after this process was put at +4 °C to be used (Antony et al., 2013).

2.2. Biological synthesis of AgNPs by green synthesis method

The dried plant leaves were crushed in a mortar and powdered, 10 g were weighed, and distilled water was added to it. The magnetic heater was also subjected to the extraction process for 4-5 hours, and at the end of the period, filtering was done using filter papers. 60 ml of the prepared extract was taken, and 1 mM silver nitrate ($AgNO_3$) solution was added. The mixture was stirred in the magnetic heater for 1 hour until a color change occurred. After the color change was observed, the mixture was centrifuged at 13.000 rpm for 15 minutes, and thus the nanoparticles were purified from other organic molecules. Each time, the supernatant was poured and added from the prepared solution again. Finally, the part remaining at the bottom was dried in an oven at 35 °C (Rather et al., 2013).

2.3. Characterization of synthesized AgNPs

Many methods are used to characterize nanoparticles. In this study, SEM, UV-Vis, and XRD, the basic techniques for the characterization of AgNPs, were used. The analyzes were made by the Eastern Anatolia High Technology Center (DAYTAM), which operates within Atatürk University.

2.3.1. SEM analysis

The characterization of the shape, surface topology, and morphology of the obtained AgNPs was carried out using the Zeiss Sigma 30 model scanning electron microscope (SEM) located at Eastern Anatolia High Technology Center (DAYTAM) Ataturk University.

2.3.2. UV-Vis spectroscopy

1 mg of AgNPs was dissolved in 3 ml of deionized water. The sample was sonicated for a certain time for the AgNPs to show a good distribution in water. This solution was then placed in the cuvette, and the AgNPs were characterized by measuring their UV-Vis spectra at wavelengths between 200 and 875 nm.

2.3.3. XRD analysis

The XRD pattern of the synthesized AgNPs was analyzed with the PANalytical Empyrean XRD device with a step size of 0.02 in the range of 2θ between 10° and 90°. UV-Vis spectra and XRD results were evaluated in the OriginLab data analysis program, and their graphics were drawn.

2.4. Determination of antibacterial activities

2.4.1. Microorganisms used in the study

A total of 6 strains of gram-positive [*Bacillus subtilis* (DSMZ 1971), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212)] and gram-negative [*Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13075), *Escherichia coli* (ATCC 25922)] microorganisms selected by using minimum inhibition concentration (MIC) method. As positive controls, Ertapenem, Tetracycline, Gentamicin (10 mg/ml) antibiotic discs were used.

2.4.2. Disc diffusion method

Sterile, disposable, MH (Mueller Hinton) agar with a 4 mm height medium was used on petri dishes with a diameter of 15 cm. A sterile loop was taken from the bacterial colonies that grew as pure colonies on the culture plates and inoculated into MH broth, incubated for 1-2 hours at 37 °C. After turbidity was formed, standard turbidity was established by adjusting McFarland 0.5 (10^8 microorganisms/ml). Widespread cultivation was performed from this suspension on MH agar medium with a sterile swab. Dilutions of the substance to be investigated with distilled water at a certain concentration were prepared. Paper disks impregnated with 20 µl of these dilutions were placed in the medium with sterile forceps. 3 were run in parallel. Three different antibiotics [Ertapenem, Tetracycline, and Gentamicin (10 mg/ml)] were used for control purposes. After incubation of petri dishes at 35-37 °C for 18-24 hours, inhibition zone diameters were measured (Hudzicki, 2009).

2.5. MIC analysis

The antibacterial activities of silver nanoparticles were determined by the minimum inhibition concentration (MIC) method (Wang et al., 2017). For the microdilution method, 96-well microplate wells were used. In applications carried out with the microdilution method, an MHB medium for bacteria was added to the wells of 96 microplates. After preparing a series of dilutions from the adjusted concentrations of silver nanoparticles, AgNPs solutions were added to the microplates and diluted. Then, a certain amount of microorganism solutions prepared and adjusted according to 0.5 McFarland (1.5×10^8 CFU/ml) were added to the microplates and

incubated overnight at 37 °C for 24 hours. At the end of the period, the absorbance value was measured at 600 nm in the spectrophotometer device. Obtained results were schematized as multiples of MIC, and % inhibition was calculated.

2.6. Methods applied in Determining antioxidant capacity

2.6. Identification of radical scavenging potential

2.6.1. DPPH method

To prepare a 0.1 mM DPPH solution, 4 mg of DPPH was weighed. It was dissolved by adding 200 ml of methanol. 50µl of extract and AgNPs sample was added to eppendorf tubes. It was vortexed by adding 200µl of DPPH solution to them. The absorbance was measured at 517 nm after being kept in the dark for 60 minutes and added to the 96 well plate wells (Almeida et al., 2020).

2.6.2. ABTS method

To prepare a 7 mM ABTS solution, 0.192 g ABTS and 0.0324 g potassium persulfate were added to 50 ml distilled water. The volume was completed to 100 ml and kept in the dark overnight by mixing these mixtures. Then, 1 ml of this mixture was taken, and 39 ml of methanol was added to it and diluted to 40 ml. After dilution, 15µl of plant extract and AgNPs were taken. 285 µl of ABTS solution was added to them and vortexed. After 2 hours in the dark, absorbance was measured at 734 nm (Almeida et al., 2020).

2.7. Lipoxygenase enzyme inhibition

In the determination of lipoxygenase activity of the samples, the spectrophotometric method is given by Anthon and Barrett (2012) was used with some modifications. To determine the lipoxygenase activity, 0.2% Triton X-100 was added to 0.1 M phosphate buffer (pH 6.5) to free the enzyme, and a homogenization buffer was prepared. Linoleic acid was used as a substrate for the activity assay. To prepare 25 ml of the linoleic acid substrate, 280 mg of Tween 20 and 140 mg of linoleic acid were added to 5 ml of distilled water. 0.6 ml, 1 N NaOH was added to clarify the solution. The volume was made up to 25 ml with distilled water and stored at -18 °C as 1 ml aliquots. At the extraction stage, approximately 5 g of the sample was weighed, and the same amount of cold (4 °C) homogenization buffer was added and centrifuged at 9000 g for 10 minutes. After centrifugation, some of the supernatants were transferred to a clean centrifuge tube. The enzyme obtained from soybean [Glycine max (L.) Merr.] was used. The enzyme solution was prepared by weighing 5 mg enzyme and dissolving it in a 4.3 ml phosphate buffer. The prepared enzyme solution was stored in eppendorf tubes in 250 µl portions at -80 °C until the experiments were carried out. Nordihydroguaiaretic acid (NDGA) was used as the reference inhibitor. 2 mg of NDGA was weighed and first dissolved in 2 ml of methanol. Pure water, buffer solution, substrate solution, and inhibitor mixture was used as blank. The reaction started with the addition of 0.2 ml of the enzyme extract, and the increase in absorbance at 234 nm wavelength was observed for 3 minutes. The amount of enzyme was calculated as nmol/GFW/min from the slope of the absorbance curve at 234 nm wavelength versus time.

3. Results and discussion

3.1. Characterization of AgNPs

3.1.1. SEM results

SEM images of AgNPs obtained from *S. resinosum* by the green synthesis method are given in Figure 1. From the SEM images, it is seen that the particles have different diameters and sizes. SEM images of AgNPs were evaluated, and they were found to be spherical. With the *Trianthema decandra* extract, it was determined that the particles were 33 nm on average (Geethalakshmi and Sarada, 2010). They synthesized spherical nanoparticles with sizes ranging from 16-50 nm with *Sterculia foetida* extract (Premkumar et al., 2018). In another study with orchid extract, spherical-looking AgNPs varying between 15-40 nm were obtained (Gopinath et al., 2017).

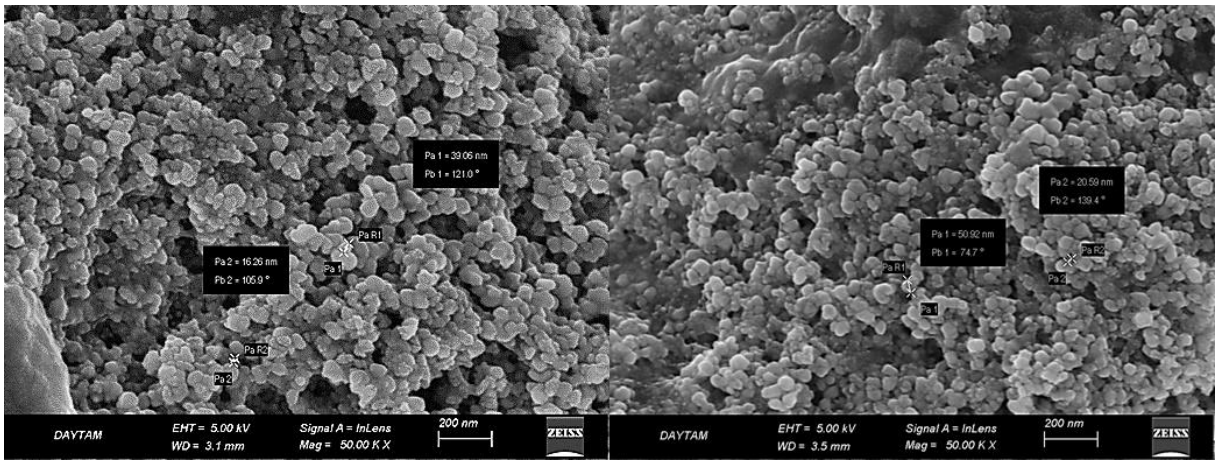

Figure 1. Images of SEM analyzes of synthesized silver nanoparticles

3.1.2. UV-Visible absorption analysis

The formation of silver nanoparticles causes vibrations on the plasma surface to change the color of the solution from yellow to brown. This indicates the formation of nanoparticles (Geethalakshmi and Sarada, 2010; Al-Ogaidi et al., 2017). The color change observed in the study and the data with a maximum absorbance of 420 nm in UV-Vis measurements supports this formation (Figure 2). Similar absorbances are observed in the study with green coffee (Wang et al., 2017). Alsammarraie et al. (2018) stated that they found a maximum value of 435 nm due to UV-Vis analysis using turmeric (*Curcuma longa*) plant extract. In another study, the maximum

absorbance of 461 nm was determined in the synthesis of AgNPs with the plant extract of corn leaves (Eren and Baran, 2019).

3.1.3. XRD analysis

The X-ray diffraction of the obtained AgNPs was evaluated (Figure 3), and the characteristic peaks of silver at 111, 200, 220, and 311 (with values of 27.81o, 32.22o, 46.27o and 54.89o at 2θ) showed the crystal structure of silver. It was observed that similar data were obtained due to the synthesis made with *Sida cordifolia* plant extract (Pallela et al., 2018).

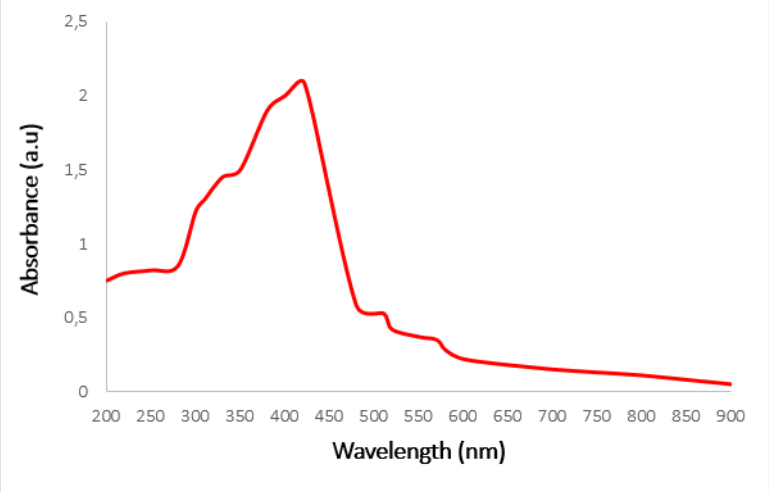

Figure 2. UV-Vis spectrum of synthesized AgNPs

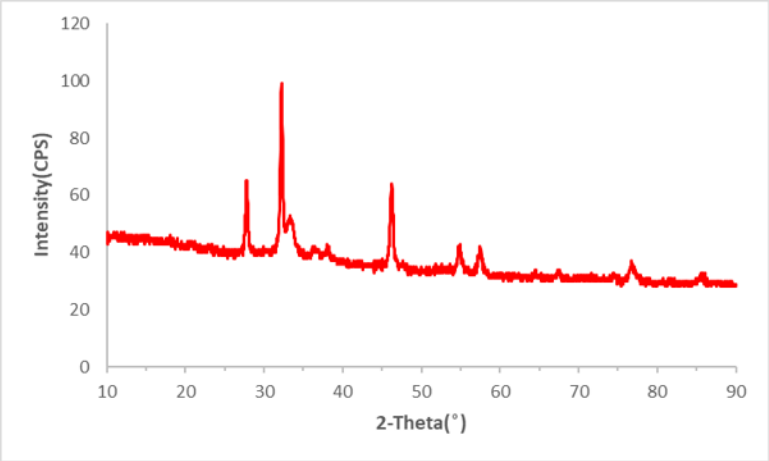

Figure 3. XRD results of synthesized AgNPs

3.2. Antibacterial activity results

In this study, antibacterial activities of AgNPs were obtained using *S. resinosum* plant; disc diffusion was evaluated against a total of 6 strains of gram-positive (*B. subtilis*, *S. aureus*, *E. faecalis*) and gram-negative (*P. aeruginosa*, *S. enteritidis*, *E. coli*) microorganisms selected by minimum inhibition concentration (MIC) methods. As positive controls, Ertapenem, Tetracycline, Gentamicin (10 mg/ml) antibiotic discs were used. It was determined that the antibiotics showed an inhibition zone in the range of 7-13 mm against the test microorganisms. Disc diameters were included when calculating the results. The minimum inhibitory concentration value of the plant extract and AgNPs showing antibacterial effect by the disc diffusion method was determined using 96-well microplates. After the incubation period was complete, the spectrophotometer (600nm) was measured. The minimum inhibitory values (mg/ml) of the tested compounds against bacteria are given in Table 1. The MIC range for *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *B. subtilis* DSMZ 1971, *E. faecalis* ATCC 29212, and *S. enteritidis* ATCC 13075 was between 0.625-0.001 mg/ml, and the MIC_{90} values were 0.009, 0.009, 0.019, 0.019, 0.019, and 0.009 mg/ml, respectively. 95.83% of *E. coli* (ATCC 25922), 94.76% of *P. aeruginosa* (ATCC 27853), 84.14% of *S. aureus* (ATCC 25923), 83.07% of *B. subtilis* (DSMZ 1971), 80.93% of *E. faecalis* (ATCC 29212), and 92.62% of *S. enteritidis* (ATCC 13075) were found to be sensitive to the substance (Table 1). Dipankar and Murugan (2012) reported that silver nanoparticles obtained from *Iresine herbstii* leaf extract showed strong antibacterial activity against *S. aureus*, *P. aeruginosa*, *E. coli*, *E. faecalis*, and *Klebsiella pneumoniae* bacteria. Singhal et al. (2011) analyzed the AgNPs obtained from the *Ocimum sanctum* plant extract, a basil species, on *E. coli* and *S. aureus* bacteria and stated that nanoparticles had an antibacterial effect even at lower concentrations than standard antibiotics. According to the results obtained, AgNPs showed bacteriocidal effects against all bacteria. It was observed that the nanoparticles we used in our study also showed antibacterial effects at low concentrations. They

reported that smaller-sized AgNPs might cause more toxicity in bacteria, and they may have a better bactericidal effect than larger particles because they have a larger surface area (Zhang et al., 2016). Agnihotri et al. (2014) found that AgNPs smaller than 10 nm increased their antibacterial activity. Banala et al. (2015) reported that a 25 µg/ml concentration of AgNPs obtained with *Carica papaya* leaf extract has the minimum inhibition concentration for gram-positive and gram-negative bacteria. It was observed that the nanoparticles we used in our study also showed antibacterial effects at low concentrations (Table 2).

Table 1. MIC ranges, MIC_{90} values, and % inhibition values of the chemical according to different species

Bacterial isolates	MIC range (mg/ml)	MIC_{90} (mg/ml)	% Inhibition
E. coli	0.625-0.001	0.009	95.83
P. aeruginosa	0.625-0.001	0.009	94.76
S. aureus	0.625-0.001	0.019	84.14
B. subtilis	0.625-0.001	0.019	83.07
E. faecalis	0.625-0.001	0.019	80.93
S. enteritidis	0.625-0.001	0.009	92.62

Table 2. Zone diameters of AgNPs in mm against bacterial strains by disc diffusion method*

Bacterial isolates	Amount(mg/ml)	Zone diameters (mm)	Control antibiotic zone diameters		
			Ertapenem	Tetracycline	Gentamicin
S. aureus	5.0	13 ± 0	28 mm	34 mm	25 mm
	2.5	12.33 ± 0.57			
	1.25	12 ± 0			
	0.625	11 ± 0			
E. coli	5.0	11.33 ± 0.57	35mm	28 mm	25 mm
	2.5	10.66 ± 0.57			
	1.25	10 ± 0			
	0.625	10 ± 0			
P. aeruginosa	5.0	12 ± 0	19 mm	16 mm	25 mm
	2.5	11.66 ± 0.57			
	1.25	10.66 ± 0.57			
	0.625	10.66 ± 0.57			
B. subtilis	5.0	11.5 ± 0.57	25 mm	18 mm	25 mm
	2.5	11 ± 0			
	1.25	11 ± 0			
	0.625	10.66 ± 0.57			
E. faecalis	5.0	7 ± 0	11 mm	16.5 mm	25 mm
	2.5	9.3 ± 0.57			
	1.25	10 ± 0			
	0.625	10 ± 0			
S. enteritidis	5.0	13 ± 0	19 mm	14.5 mm	25 mm
	2.5	12.6 ± 0.57			
	1.25	12 ± 0			
	0.625	11.33 ± 0.57			

*In the table, the inhibition zone diameters are given in mm and the mean ± SE.

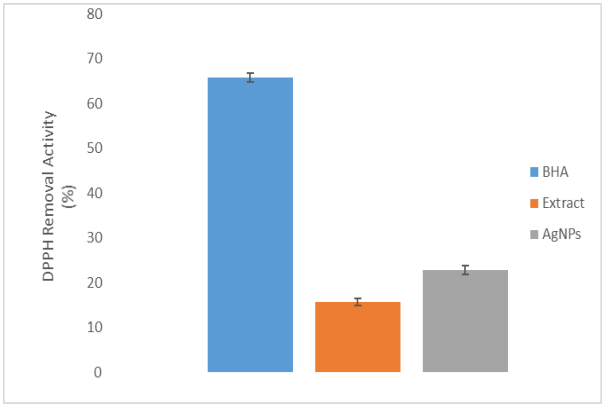

Figure 4. Plant extract and AgNPs DPPH removal activity (%)

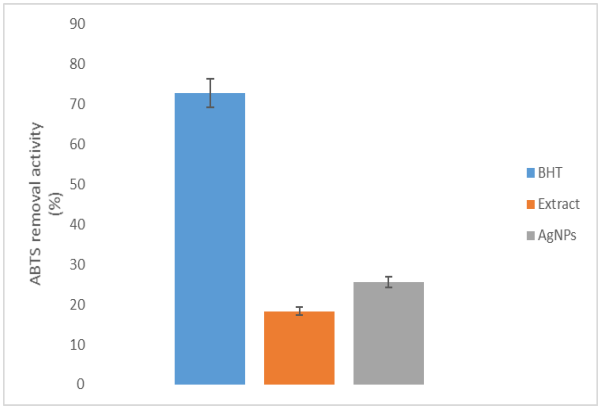

Figure 5. Plant extract and AgNPs ABTS removal activity(%)

3.3. Results of antioxidant capacity tests

DPPH (2,2-diphenyl-1-picrylhydrazil) is organic nitrogen radical commercially available product. It is a simple and fast method used to measure the antioxidant capacity of plant extracts. In DPPH removal activity, AgNPs obtained by green synthesis provided a high percentage of inhibition removal than extract. Silver nanoparticles provided 22% removal based on this percentage, while extract caused 15% removal (Figure 4). It shows that the flavonoid and phenolic groups in *S. resinosum* have a synergistic effect on the silver nanoparticle, and besides, the physical and chemical properties of the silver nanoparticle were also effective in DPPH removal. DPPH removal activity of butylated hydroxyl anisole (BHA) was 65%, and it was higher than extract and silver nanoparticle obtained by green synthesis. Silver nanoparticles obtained by green synthesis provided 25% ABTS removal. The extract showed an 18%

removal rate (Figure 5). BHT (Butylated hydroxytoluene) provided 72% ABTS removal compared to the nanoparticle and extract obtained by the biological method. According to the results, it was observed that the best ABTS removal was in the nanoparticle obtained by biological synthesis (25%). This value is compatible with previous studies (Genc, 2021; Genc et al., 2020), and the free radical scavenging power of silver nanoparticles is related to different types of functional groups responsible for the reduction and coating of silver nanoparticles. Our results showed that these biological components increased the antioxidant activity of silver nanoparticles.

Table 3. Lipoxygenase inhibition of plant extract and AgNPs

Plant Name	Extract/AgNPs	LOX inhibition (IC_{50} µg/mL)
S. resinosum	Extract	6.83 ± 1.1
	AgNPs	3.67 ± 0.5

3.4. Results of lipoxygenase enzyme inhibition

The inhibition potential of aqueous and methanolic extracts of *S. resinosum* on LOX enzyme was measured *in vitro*. NDGA was used as standard. Concentrations of 1, 1.3, 1.6, 2.3, and 2.6 µg/mL of NDGA have been tested. The NDGA substance inhibited the LOX enzyme by 66.6%, even at a 1 µg/mL concentration. The concentration showing the IC_{50} value of NDGA (which inhibits the enzyme by 50%) was calculated as 1.09 µg/mL. The effect of extract and AgNPs of plants on lipoxygenase activity is shown in Table 3. *S. resinosum* extract and AgNPs inhibited LOX less than NDGA (Figures 6, 7, and 8). *S. resinosum* was found to have LOX inhibitory activity. The IC_{50} value of the AgNPs was 3.67 ± 0.5 mg/ml, and the IC_{50} value of the extract was 6.83 ± 1.1 µg/ml. The higher inhibitory activity is associated with a lower IC_{50} value. This plant extract is the most active compound against the enzyme. Inhibition of lipoxygenase enzyme activity by various plants has been studied. They showed that *Epilobium angustofolium* extract inhibited lipoxygenase activity, and they found the IC_{50} value as 0.57 ± 0.06 µg/ml (Onar et al., 2012). Water extract of *Pituranthos chloranthus* showed strong lipoxygenase inhibition with an IC_{50} value of 0.02 mg/ml. Soybean lipoxygenase was successfully inhibited by *Lavatera cretica* leaf and flower water extracts with an IC_{50} value of 0.01 µg/ml (Lončarić et al., 2021). The results suggest that plant extracts have a potentially high anti-inflammatory effect (antilipoxygenase activity), related to polyphenolic content and other antioxidant substances.

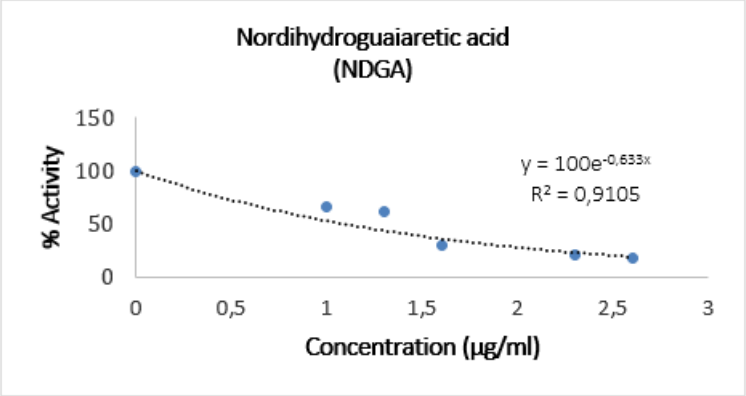

Figure 6. The IC_{50} value of NDGA to LOX enzyme

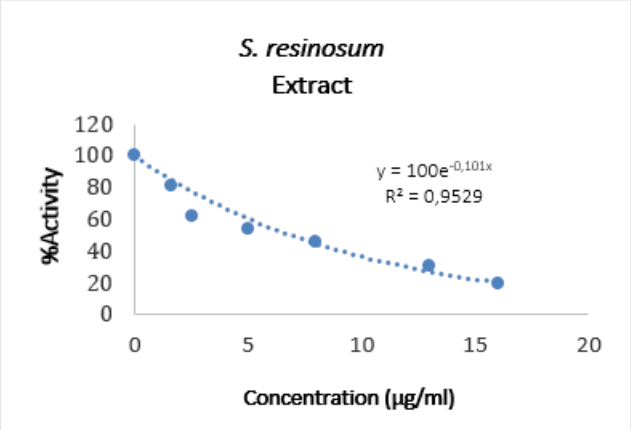

Figure 7. The IC_{50} value of plant extract to LOX enzyme

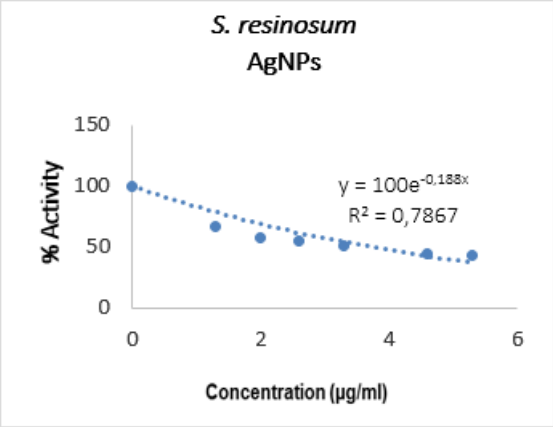

Figure 8. The IC_{50} value of AgNPs to LOX enzyme

4. Conclusions

The interest in the green synthesis method used to obtain nanoparticles increases day by day. It has been determined that these particles are effective at lower concentrations against commercial antibiotics, and AgNPs synthesized by the green method has a good antibacterial effect. With the increase in nanotechnological research, synthesized AgNPs will open a new field in producing pharmaceutical products; in the pharmaceutical industry, biomedical and industrial products can become more useful. Also, in this study, it has been shown that *S. resinosum* extract and AgNPs synthesized from the extract have LOX inhibitory

activity, indicating that they may be useful in treating various inflammatory diseases such as cancer, allergic disease, asthma, aging, and atherosclerosis. In addition, the biochemical examination of the effect of the plant extracts on the inhibition of target enzymes may pave the way for their presentation to the biotechnology market as alternative drug molecules to be used in different fields of pharmaceutical chemistry and industry.

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None.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ozlem Bakir: Conceptualization, Investigation, Data curation, Writing - original draft, Visualization
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Supplementary File

None.

References

Agnihotri, S., Mukherji, S., Mukherji, S., 2014. Size-controlled silver nanoparticles synthesized over the range 5-100 nm using the same protocol and their antibacterial efficacy. *RSC Advances*, 4(8), 3974-3983.

Ahmed, S., Ahmad, M., Swami, B.L., Ikram, S., 2016. A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: a green expertise. *Journal of Advanced Research*, 7(1), 17-28.

Almeida, A.D.R., Maciel, M.V.D.O.B., Machado, M.H., Bazzo, G.C., de Armas, R.D., Vitorino, V.B., Barreto, P.L.M., 2020. Bioactive compounds and antioxidant activities of Brazilian hop (*Humulus lupulus* L.) extracts. *International Journal of Food Science & Technology*, 55(1), 340-347.

Al-Ogaidi, I., Salman, M.I., Mohammad, F.I., Aguilar, Z., Al-Ogaidi, M., Hadi, Y.A., Abd Al-Rhman, R.M., 2017. Antibacterial and cytotoxicity of silver nanoparticles synthesized in green and black tea. *World Journal of Experimental Biosciences*, 5(1), 39-45.

Alsammarraie, F.K., Wang, W., Zhou, P., Mustapha, A., Lin, M., 2018. Green synthesis of silver nanoparticles using turmeric extracts and investigation of their antibacterial activities. *Colloids and Surfaces B: Biointerfaces*, 171, 398-405.

Anthon, G.E., Barrett, D.M., 2012. Pectin methylesterase activity and other factors affecting pH and titratable acidity in processing tomatoes. *Food Chemistry*, 132(2), 915-920.

Antony, J.J., Nivedheetha, M., Siva, D., Pradeepha, G., Kokilavani, P., Kalaiselvi, S., Achiraman, S., 2013. Antimicrobial activity of *Leucas aspera* engineered silver nanoparticles against *Aeromonas hydrophila* in infected *Catla catla*. *Colloids and Surfaces B: Biointerfaces*, 109, 20-24.

Banala, R.R., Nagati, V.B., Karnati, P.R., 2015. Green synthesis and characterization of *Carica papaya* leaf extract coated silver nanoparticles through X-ray diffraction, electron microscopy and evaluation of bactericidal properties, Saudi Journal of Biological Sciences, 22(5), 637-644.

Chen, J.C., Lin, Z.H., Ma, X.X., 2003. Evidence of the production of silver nanoparticles via pretreatment of *Phoma* sp. 3.2883 with silver nitrate. *Letters in Applied Microbiology*, 37(2), 105-108.

Davis, P.H., 1970. Flora of Turkey and the East Aegean Islands. Vol. 3. Flora of Turkey and the East Aegean Islands. Vol. 3.

Dipankar, C., Murugan, S., 2012. The green synthesis, characterization and evaluation of the biological activities of silver nanoparticles synthesized from *Iresine herbstii* leaf aqueous extracts. *Colloids and Surfaces B: Biointerfaces*, 98, 112-119.

Eren, A., Baran, M.F., 2019. Green synthesis, characterization and antimicrobial activity of silver nanoparticles (AgNPs) from maize (*Zea mays* L.). *Applied Ecology and Environmental Research*, 17(2), 4097-4105.

Francis, S., Joseph, S., Koshy, E.P., Mathew, B., 2017. Green synthesis and characterization of gold and silver nanoparticles using *Mussaenda glabrata* leaf extract and their environmental applications to dye degradation. *Environmental Science and Pollution Research*, 24(21), 17347-17357.

Geethalakshmi, R., Sarada, D.V.L., 2010. Synthesis of plant-mediated silver nanoparticles using *Trianthema decandra* extract and evaluation of their anti microbial activities. *International Journal of Engineering Science and Technology*, 2(5), 970-975.

Genc, N., 2021. Biosynthesis of silver nanoparticles using *Origanum onites* extract and investigation of their antioxidant activity. *Particulate Science and Technology*, 39(5), 562-568.

Genc, N., Yildiz, I., Chaoui, R., Erenler, R., Temiz, C., Elmastas, M., 2020. Biosynthesis, characterization and antioxidant activity of oleuropein-mediated silver nanoparticles. *Inorganic and Nano-Metal Chemistry*, 51(3), 411-419.

Gopinath, K., Devi, N.P., Govindarajan, M., Bhakyaraj, K., Kumaraguru, S., Arumugam, A., Benelli, G., 2017. One-Pot green synthesis of silver nanoparticles using the orchid leaf extracts of *Anoectochilus elatus*: growth inhibition activity on seven microbial pathogens. *Journal of Cluster Science*, 28(3), 1541-1550.

Gopinath, K., Kumaraguru, S., Bhakyaraj, K., Mohan, S., Venkatesh, K.S., Esakkirajan, M., Arumugam, A., 2016. Green synthesis of silver, gold and silver/gold bimetallic nanoparticles using the *Gloriosa superba* leaf extract and their antibacterial and antibiofilm activities. *Microbial Pathogenesis*, 101, 1-11.

Guzel, A., Aksit, H., Elmastas, M., Erenler, R., 2017. Bioassay-guided isolation and identification of antioxidant flavonoids from *Cyclotrichium origanifolium* (Labill.) manden and scheng. *Pharmacognosy Magazine*, 13(50), 316-320.

Halliwell, B., 1990. How to characterize a biological antioxidant. *Free Radical Research Communications*, 9(1), 1-32.

Han, H., Yılmaz, H., Gulcin, I. 2018. Antioxidant activity of flaxseed (*Linum usitatissimum* L.) and analysis of its polyphenol contents by LC-MS/MS. *Records of Natural Products*, 12(4),397–402.

Hudzicki, J., 2009. Kirby-Bauer disk diffusion susceptibility test protocol. *American Society for Microbiology*, 15, 55-63.

Juntachote, T., Berghofer, E., 2005. Antioxidative properties and stability of ethanolic extracts of Holy basil and Galangal. *Food Chemistry*, 92(2), 193-202.

Kelavkar, U.P., Cohen, C., Kamitani, H., Eling, T.E., Badr, K.F. 2000. Concordant induction of 15-lipoxygenase-1 and mutant p53 expression in human prostate adenocarcinoma: correlation with Gleason stating. *Carcinogenesis*, 21,1777-1787.

Kung, J.C., Chen, Y.J., Chiang, Y.C., Lee, C.L., Yang-Wang, Y.T., Hung, C.C., Shih, C.J., 2018. Antibacterial activity of silver nanoparticle (AgNP) confined mesoporous structured bioactive powder against *Enterococcus faecalis* infecting root canal systems. *Journal of Non-Crystalline Solids*, 502(15), 62-70.

Lončarić, M., Strelec, I., Moslavac, T., Šubarić, D., Pavić, V., Molnar, M., 2021. Lipoxygenase inhibition by plant extracts. *Biomolecules*, 11(2), 152.

Onar, H.C., Yusufoglu, A., Turker, G., Yanardag, R., 2012. Elastase, tyrosinase and lipoxygenase inhibition and antioxidant activity of an aqueous extract from *Epilobium angustifolium* L. leaves. *Journal of Medicinal Plants Research*, 6(5), 716-726.

Pallela, P.N.V.K., Ummey, S., Ruddaraju, L.K., Pammi, S.V.N., Yoon, S.G., 2018. Ultra Small, mono dispersed green synthesized silver nanoparticles using aqueous extract of *Sida cordifolia* plant and investigation of antibacterial activity. *Microbial Pathogenesis*, 124, 63-69.

Premkumar, J., Sudhakar, T., Dhakal, A., Shrestha, J.B., Krishnakumar, S., Balashanmugam, P., 2018. Synthesis of silver nanoparticles (AgNPs) from cinnamon against bacterial pathogens. *Biocatalysis and Agricultural Biotechnology*, 15, 311-316.

Rather, M.A., Sharma, R., Gupta, S., Ferosekhan, S., Ramya, V.L., Jadhao, S.B., 2013. Chitosan-nanoconjugated hormone nanoparticles for sustained surge of gonadotropins and enhanced reproductive output in female fish. *PloS one*, 8(2), e57094.

Samuelsson, B., Dahlen, S.E., Lindgren, J.Å., Rouzer, C.A., Serhan, C.N., 1987. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science*, 237(4819), 1171-1176.

Saravanakumar, K., Chelliah, R., Shanmugam, S., Varukattu, N.B., Oh, D.H., Kathiresan, K., Wang, M.H., 2018. Green synthesis and characterization of biologically active nanosilver from seed extract of *Gardenia jasminoides* Ellis. *Journal of Photochemistry and Photobiology B: Biology*, 185, 126-135.

Selvakumar, P., Sithara, R., Viveka, K., Sivashanmugam, P., 2018. Green synthesis of silver nanoparticles using leaf extract of *Acalypha hispida* and its application in blood compatibility. *Journal of Photochemistry and Photobiology B: Biology*, 182, 52-61.

Sindhi ,V., Gupta, V., Sharma, K., Bhatnagar, S., Kumari, R., Dhaka, N. 2013. Potential applications of antioxidants—a review. *Journal of Pharmacy Research*, 7,828–835.

Singhal, G., Bhavesh, R., Kasariya, K., Sharma, A.R., Singh, R.P., 2011. Biosynthesis of silver nanoparticles using *Ocimum sanctum* (Tulsi) leaf extract and screening its antimicrobial activity. *Journal of Nanoparticle Research*, 13(7), 2981-2988.

Wang, M., Zhang, W., Zheng, X., Zhu, P., 2017. Antibacterial and catalytic activities of biosynthesized silver nanoparticles prepared by using an aqueous extract of green coffee bean as a reducing agent. *RSC Advances*, 7(20), 12144-12149.

Xu, H., Shi, X., Ma, H., Lv, Y., Zhang, L., Mao, Z., 2011. The preparation and antibacterial effects of dopa-cotton/AgNPs. *Applied Surface Science*, 257(15), 6799-6803.

Yang, G., Xie, J., Deng, Y., Bian, Y., Hong, F., 2012. Hydrothermal synthesis of bacterial cellulose/AgNPs composite: a “green” route for antibacterial application. *Carbohydrate Polymers*, 87(4), 2482-2487.

Zhang, X.F., Liu, Z.G., Shen, W.,Gurunathan, S., 2016. SilverNanoparticles: Synthesis, Characterization, Properties, Applications, and Therapeutic Approaches. *International Journal of Molecular Sciences*, 17, 1534.

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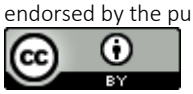

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REVIEW

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Effect of berberine on irritable bowel syndrome: A symptom-based review

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ABSTRACT

Phytotherapeutic applications have gained a place in the symptomatic treatment of irritable bowel syndrome, one of the most common diseases globally among functional bowel diseases. This study aimed to compile evidence regarding the efficacy of berberine in relieving the symptoms of irritable bowel syndrome. In this review, the electronic databases of PubMed, Google Scholar, MEDLINE, and Web of Science were used, and current publications were searched without any language restrictions. The screening was performed by first performing a general search using the keyword "berberine and irritable bowel syndrome", followed by individual searches for each symptom. Although there are many preclinical studies investigating the effect of berberine on the gastrointestinal tract, human studies are still limited. Studies show that berberine may positively affect abdominal pain, diarrhea, inflammation, microbiota, and visceral hypersensitivity, although the mechanisms of action are not yet clear. However, available data suggest that the therapeutic use of berberine in irritable bowel syndrome may be limited to the predominant type of diarrhea and selected patients whose symptoms are evaluated individually. Evidence presented in this paper needs to be further supported by human clinical studies.

1. Introduction

Irritable bowel syndrome (IBS) is a chronic functional bowel disease with an uncertain etiology and characterized by upper and lower gastrointestinal symptoms, such as chronic or recurrent fluctuating abdominal pain, changes in bowel habits (constipation/diarrhea), and dyspeptic complaints (meteorism, bloating, burping) (Longstreth et al., 2006). It has been reported that psychosocial factors, gut microbiota, diet, genetics, and inflammatory factors may play a role in the etiology of IBS (Soares, 2014). Recently, additional potential mechanisms of IBS, including gut microbiota alteration and low-grade inflammation/immune activation, have emerged, and the importance of gut microbiota in the etiology of the disease has been emphasized (Habtemariam, 2020; Longstreth et al., 2006).

Since there is still no diagnostic test or structural or biochemical markers specific to IBS, its diagnosis is made by excluding other organic diseases with similar manifestations and evaluating patient symptoms according to the Rome IV criteria. According to the Bristol stool chart scores within the Rome IV diagnostic criteria, IBS is divided into four groups as “IBS with predominant diarrhea”, “predominant constipation”, “mixed bowel habits”, and “unclassified” (IBS-D, IBS-C, IBS-M, and IBS-U, respectively) (Schmulson and Drossman, 2017). Among the functional bowel diseases, IBS is one of the most common diseases globally, with an approximate prevalence of 11.2% (Lovell and Ford, 2012; Ooi et al., 2019). Many outpatient visits, excessive examinations, unnecessary drug treatment, and the inability to obtain satisfactory results until IBS diagnosis causes a significant increase in health costs and loss of workforce (Inadomi et al., 2003; Longstreth et al., 2003).

In the symptomatic treatment of IBS, antispasmodics (otilonium bromide, mebeverine, alverine, and trimebutine) are used for abdominal pain (Clavé et al., 2011). It is known that in cases where constipation is dominant, fibrous supplements can be used in mild and moderate cases, and linaclotide and lubiprostone can relieve patients’ symptoms through their effects on meteorism, abdominal pain, constipation severity, and stool consistency (Drossman et al., 2009; Chey et al., 2012). In treating diarrheal cases, the constipating side effects of bile-binding resins, such as cholestyramine,

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antidiarrheal agents (loperamide, etc.), or tricyclic antidepressants are used (Khan and Chang, 2010; Can and Yilmaz, 2015). Complementary/alternative medicine applications are also included in the treatment of IBS, and their use rate reaches 48% (Langmead et al., 2002). Other treatment modalities include acupuncture, cognitive behavioral therapy, and hypnosis (Payne and Blanchard, 1995; Webb et al., 2007; Palsson and Whitehead, 2013).

The most problematic stage in IBS management is treatment, and a step-by-step approach is recommended (Table 1) (Mansueto et al., 2015; Akyuz, 2016; Schmulson and Drossman, 2017). It is also reported that pharmacotherapeutic agents should be avoided in asymptomatic periods (Talley and Spiller, 2002).

Table 1. Step-by-step therapy for IBS symptoms

Symptom	Treatment methods	Primary treatment	Secondary treatment
Abdominal pain	Drug therapy	Antispasmodic	TCA
			SSRI
	Complementary therapy	Peppermint oil	Acupuncture
			Hypnosis
			Psychotherapy
Abdominal distention	Drug therapy	Antispasmodic	Rifaximin
		Simethicone	Probiotics
		Activated carbon	5-HT4 agonists
		Bismuth subsalicylate	TCA
		Pancreatic enzyme replacement	
	Complementary therapy	Low-carb diet	
		Fennel Chamomile	
Diarrhea	Drug therapy	Opioid agonists	5-HT3 agonists
		Bile acid sequestrants	
		Probiotics	
		Antibiotics	
	Complementary therapy	Gluten-free diet	
		FODMAP diet	
		Fibrous foods in mild to moderate cases	5-HT4 agonists
		Laxatives (polyethylene glycol, magnesium)	Secretagogue
		Mixed opioid antagonists	Lubiprostone
Constipation	Drug therapy	Psyllium	Linaclotide
		Calcium channel activators	Prucalopride
		Guanylate cyclase C agonists	
	Complementary therapy	Fibrous supplements, senna	

TCA: Tricyclic antidepressants, SSRI: Selective serotonin reuptake inhibitors, FODMAP: The low-fermentable oligo-, di-, and monosaccharide and polyol

Active ingredients obtained from plants have been used to treat diseases since ancient times, and the transfer of herbal-derived active ingredients to modern treatment is becoming increasingly common (Kidd, 2009). With the introduction of the new complexing technique “phytosome”, it is now possible to facilitate the absorption and increase the bioavailability of plant-derived active substances with long side chains and high polarity it is possible to (Scalbert and Williamson, 2000). However, the bioavailability of such substances is reduced due to their first-pass effect in the liver and intestine. Therefore, to use berberine (BBR) in treatment, it is appropriate to use the phytosome formulation developed as a drug delivery system for this chemical (Yu et al., 2017).

Berberine ($C_{20}H_{17}ONO_5$) is a quaternary benzylisoquinoline alkaloid that can be obtained from many different plants, such as *Berberis vulgaris*, *B. aquifolium*, *Hydrastis canadensis*, *Coptis chinensis*, and it has been used in diabetes, cancer, depression, and central nervous system diseases in recent years (Kulkarni and Dhir, 2010; Liang et al., 2019; Wang et al., 2020). In addition, recent studies reported very successful results in the use of BBR in the treatment of IBS-D (Sun et al., 2014).

In this study, a review of the literature was conducted to present available evidence regarding the efficacy of BBR on IBS symptoms.

2. Materials and methods

In the research strategy of this review, the electronic databases of PubMed, Google Scholar, MEDLINE, and Web of Science were used, and all publications until August 2021 were searched without language restrictions. First, a general screening was performed with the keyword "berberine and irritable bowel syndrome", and then each symptom was searched individually using the following keywords: ''berberine and abdominal pain'', ''berberine and diarrhea'', ''berberine and visceral hypersensitivity'', ''berberine and microbiota'', ''berberine and anti-inflammatory'', and ''berberine and antinociceptive'' berberine and motility''.

3. Results and discussion

3.1. Effects of BBR on IBS symptoms

3.1.1. Abdominal pain and distention

Abdominal pain is common in IBS, and studies have shown that BBR may potentially affect visceral analgesia, particularly due to its antinociceptive effect on visceral hypersensitivity. It is considered that the analgesic effect of BBR may be related to nitric oxide and its antioxidant and anti-inflammatory activity (Hu et al., 2009; Tang et al., 2013; Kim, 2015). In the visceral pain model, BBR has been reported to reduce pain by acting on opioid receptors. BBR increased mu- and delta-opioid receptor expression in the mouse gut and rat fetal cortical neurons (Chen et al., 2015a).

In a randomized, double-blind, placebo-controlled clinical study conducted by Chen et al. (2015b), the effect of BBR hydrochloride on clinical symptoms in IBS-D was investigated. A total of 132 patients participated in the study, and two study groups were formed, with one being orally given 200 mg BBR hydrochloride (n = 70) twice a day for eight weeks (400 mg in total) or the other being designated as the placebo (n = 62). BBR hydrochloride was shown to significantly reduce the frequency of diarrhea, abdominal pain, and the need for urgent defecation. The authors also reported that they observed an improvement trend with the use of BBR hydrochloride

in the IBS symptom, depression, anxiety, and quality of life scores (Chen et al., 2015b). However, in another clinical study, gas, and meteorism were reported as common side effects of BBR, and it was suggested that these side effects might be due to the "acarbose-like" effects of this chemical on intestinal α-glucosidase (Di Pierro et al., 2020).

Table 2. Effects of BBR on IBS symptoms

Abdominal pain and distention			
Effects	**The pathway that might play a role**	**Recovery outcomes**	**Studies**
Antinociceptive	Nitric oxide	Pain threshold was significantly increased in male Sprague-Dawley rats administered BBR (50 mg/kg, i.p., once daily). Aminoguanidine reversed this effect. The BBR and BBR + aminoguanidine (nitric oxide synthetase inhibitor) groups showed reduced defecation, but aminoguanidine alone did not reduce defecation.	Tang et al. (2013)
Antinociceptive Anti-inflammatory Anti-oxidant	Myeloperoxidase and malondialdehyde activities	Berberine alleviated allodynia induced by CCI, a neuropathic pain model, and its anti-inflammatory and antioxidative properties contributed to the antiallodynic effect of CCI.	Kim et al. (2015)
Anti-inflammatory	E-selectin Thromboxane B2	Berberine downregulated E-selectin expression and decreased the content of TXB (2), effectively reducing the inflammatory response, thus relieving intestinal dysfunction via multiple pathways.	Hu et al. (2009)
Antinociceptive	Mu- and delta-opioid receptors	BBR increased mu and delta-opioid receptor expression in the mouse gut and rat fetal cortical neurons. It prolonged gastrointestinal transit and time to diarrhea reduced visceral pain.	Chen et al. (2015a)
		A randomized, double-blind, placebo-controlled human study (n = 132). Berberine hydrochloride 200 mg twice daily reduced the frequency of diarrhea, abdominal pain, and urgency to defecate.	Chen et al. (2015b)
Diarrhea			
Inhibition of gastrointestinal motility and/or visceral hypersensitivity	Upregulation of somatostatin and glucagon-like peptide-1 and downregulation of motilin and gastrin levels	BBR-based nutraceuticals (twice a day, 250 mg for 90 days) reduced diarrhea by 50-70% after 30 days and by 70 to 80% after 90 days in 39 patients with functional diarrhea or diarrhea-predominant irritable bowel syndrome	Di Pierro et al. (2020)
	Inhibition of NF-κB	Reduced smooth muscle contraction and intestinal motility and delayed intestinal transit time	Feng et al. (2013)
	Reduction of pro-inflammatory cytokine production	Reduced bowel motility Reduced ileum mucosa inflammation	Yu et al. (2019)
Anti-secretory	Decrease Cl(-) secretion	The effects of protoberberine alkaloid palmatine on active ion transport across the rat colonic epithelium were evaluated. Palmatine inhibited SK4 K(+) channels and decreased Ca(2+) activated Cl(-) secretion	Wu et al. (2008)
	Blockade of K+ channels	BBR inhibited carbachol-induced 86Rb+ efflux and K+ conductivity in the resected mucosa	Taylor et al. (1999)
Reducing epithelial permeability	Tight junctions	It produced a dose-dependent increase in transepithelial electrical resistance in Caco-2 cells.	Gu et al. (2009)
Effect on microbiota	Antimicrobial	The IC_{50}, minimum inhibitory concentration, minimum microbicidal concentration, and minimum microbiostatic concentration of BBR were evaluated, and the sensitivity was reported in the following order: *S. aureus* > *P. aeruginosa* S (sensitive) > *E. coli* S > *P. aeruginosa* R (resistant) > *E. coli* R > *B. subtilis* > *Z. ramigera* > *C. albicans* > *S. cerevisiae*	Čerňáková and Košťálová (2002)
		It reduced the growth and adhesion of *E. coli* (90% reduction in adhesion).	Sun et al. (1988)
		Berberine hydrochloride (100 mg, four times daily) reduced stool volume with diarrhea and stool cyclic adenosine monophosphate concentration by 77% in patients with cholera.	Khin et al. (1985)
		A single oral dose of 400 mg of berberine reduced mean stool volume after treatment in patients with *E. coli* infection and cholera.	Rabbani et al. (1987)
	Fecal microbiota (*Faecalibacterium* and *Bifidobacterium*)	Partially reversed Kupfer cell hyperplasia and *Faecalibacterium* by modulating the gut microbiome composition.	Jia et al. (2019)
Anti-inflammatory	Inhibition of cytokines	Inhibited IL-8 production in colonic epithelial cells Downregulated TNF, IFN-γ, KC, and IL-17 in wild-type C57BL/6 mice Significantly suppressed TNF-α elevation in colitis induced by TNBS	Zhou and Mineshita (2000) Yan et al. (2012) Zhang et al. (2011)
	Stimulation of AMP-activated protein kinase	The inhibitory effects of BBR on proinflammatory responses were eliminated by AMPK inhibition	Jeong et al. (2009)
	Mitogen-activated protein kinase pathway	In infected macrophages, BBR deactivated ERK1/2, resulting in the time-dependent activation of p38 MAPK.	Saha et al. (2011)
	Activation of the PPARγ pathway	Inhibited the overexpression of inducible COX-2 in the ileal mucosa	Feng et al. (2012)
Constipation			
Adverse events	Antidiarrheal activity	Mild to moderate constipation was the most frequently reported side effect	Yin et al. (2008), Zhang et al. (2008), Zhang et al. (2019), Chen et al. (2020)

BBR: Berberine, IBS:İrritable bowel syndrome, CCI: Chronic constriction injury, TXB (2): Thromboxane B2, NF-κB: Nuclear Factor kappa B, TNF: Tumor necrosis factor, IFN-γ:Interferon gamma, IL-17: Interleukin 17

3.1.2. Diarrhea

Many studies have shown that BBR can prevent diarrhea with different mechanisms of action. In addition to its antimicrobial effect, studies show that it plays a role in ion transport, especially in secretory diarrhea. BBR strengthens tight junctions in the Caco-2 cell line, reducing intestinal epithelial permeability and significantly increasing transepithelial electrical resistance. It also reduces epithelial permeability in the intestine (Khin et al., 1985; Rabbani et al., 1987; Sun et al., 1988; Taylor et al., 1999; Wu et al., 2008; Gu et al., 2009).

In a retrospective clinical study conducted by Di Pierro et al. (2020), 39 patients with functional diarrhea or IBS-D that used a BBR-based nutraceutical (twice a day, 250 mg for 90 days) were evaluated. It was reported that after 30 days of BBR use, diarrhea was reduced by 50-70%, and this rate of decrease reached 70 to 80% at the end of 90 days. The study results confirmed the antidiarrheal properties of BBR and provided evidence for its use in some functional intestinal diseases characterized by the frequent defecation of mushy and/or watery stool (Di Pierro et al., 2020).

3.1.2.1. Gastrointestinal motility and/or visceral hypersensitivity

BBR has been shown to significantly reduce smooth muscle contraction and intestinal motility and delay intestinal transit time. The inhibitory effect of BBR has been potentially explained by the upregulation of somatostatin and glucagon-like peptide-1 and the downregulation of motilin and gastrin levels (Feng et al., 2013).

In a mouse study conducted to demonstrate the mechanism of action of BBR, mice with IBS and intestinal inflammation were orally administered BBR, which was shown to reduce intestinal mucosal inflammation by inducing nuclear factor kappa-B (NF-κB). BBR was reported to decrease the expression of pro-inflammatory cytokines [interleukin (IL)-1β, IL-6, interferon-γ, and tumor necrosis factor-α] and promote the expression of anti-inflammatory cytokines (IL-10 and transforming growth factor-β). It was also noted to improve terminal ileum tissue inflammation and reduce intestinal motility (Yu et al., 2019).

In a randomized study on 50 mice with IBS and intestinal inflammation, the effect of BBR on visceral hypersensitivity was investigated. The authors reported that the group given BBR had improved visceral hypersensitivity compared to the placebo group, and it was suggested that this might have been mediated by nitric oxide (Tang et al., 2013).

3.1.2.2. Microbiota

Symbiont and pathobiont compositions show a balanced distribution in the microbiota of healthy individuals (Round and Mazmanian, 2009). Healthy nutrition and probiotics can positively affect the colon microbiota (Delzenne and Bindels, 2019). Studies have shown that one of the most important effects of BBR is its ability to alter gut microbiota composition. Similar to probiotics, including *Bifidobacterium adolescentis* and *Lactobacillus acidophilus*, it has been reported that BBR can induce cell death in harmful intestinal bacteria while increasing the composition of beneficial bacteria (Čerňáková and Košťálová, 2002). In addition, in another study investigating the effects of the IBS-D gut microbiome on the liver, it was emphasized that IBS-D was not only a functional bowel disease but could also cause inflammation in the liver. It has also been found that BBR can partially reverse Kupffer cell hyperplasia and inflammation in the liver by modulating the gut microbiome composition (Jia et al., 2019).

3.1.2.3. Anti-inflammatory effects

Many studies show that BBR can positively affect colitis (Zhou and Mineshita, 2000; Yan et al., 2012) and must provide evidence that the anti-inflammatory mechanism of action is mainly through inhibiting cytokine pathways. Zhou and Mineshita (2000) reported that BBR inhibited IL-8 production in colonic epithelial cells by suppressing IFN- γ and IL-17 production (Zhou and Mineshita, 2000). In addition, different studies have demonstrated that BBR inhibits the expression of proinflammatory cytokines (IL-1β, IL-6, and TNF) and may indirectly affect inflammatory pathways mediated by MAPK, AP-1, TLR4, and NF-κB (Jeong et al., 2009; Saha et al., 2011; Zhang et al., 2011; Yan et al., 2012). Moreover, BBR is a potent inhibitor of inducible COX-2, reducing COX-2 levels and attenuating colitis *in vivo* (Feng et al., 2012).

3.1.3. Constipation

The antidiarrheic effects of BBR have limited use in IBS cases with the predominant type of diarrhea (Sun et al., 2014). In clinical studies on BBR in different patient populations, mild to moderate constipation is the most frequently reported side effect (Yin et al., 2008). In a clinical study in which 116 patients with type 2 diabetes with dyslipidemia were randomized to the BBR and placebo groups, five participants in the BBR group (1.0 g daily) experienced mild to moderate constipation (Zhang et al., 2008). In another study that systematically evaluated the efficacy and safety of BBR for the treatment of hyperlipidemia, the rate of constipation was reported to be higher in the BBR group than in the control group (Zhang et al., 2019). Similarly, in another clinical research investigating the clinical potential and safety of BBR in the prevention of colorectal adenoma recurrence, constipation was determined to be the most common adverse event (1% in the BBR group and <0.5% in the placebo group) (Chen et al., 2020) (Table 2).

Although there are many preclinical studies investigating the effect of BBR on the gastrointestinal tract, human studies are still limited. Current studies have shown that BBR may positively affect abdominal pain, diarrhea, inflammation, microbiota, and visceral hypersensitivity. However, the mechanisms of action of this chemical have not yet been clarified. BBR has a wide spectrum of effects on the gastrointestinal tract, and in this review, its effect on IBS was evaluated by considering each symptom separately. Studies show that the use of BBR in IBS may be limited only to the predominant type of diarrhea, and constipation appears as a side effect. Although many preclinical studies report that BBR reduces abdominal pain, another symptom of IBS, and has an antinociceptive effect, it should be used with caution, especially in patients with distention.

4. Conclusions

Existing studies provide evidence for the use of BBR as a drug in IBS-D and show that it can be beneficial when used in selected patients. However, the evidence presented in this paper needs to be supported by further human studies to evaluate the efficacy and side-effect profile at different doses, examine the interaction with other drugs, and investigate the effects on other comorbidities.

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None.

Conflict of interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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References

Akyuz, F., 2016. İrritabl Barsak Sendromu. *Güncel Gastroenteroloji Dergisi*; 20(4), 415-420.

Can, G., Yılmaz, B., 2015. İrritabl bağırsak sendromunun tanı ve tedavisinde yaklaşımlar. *Güncel Gastroenteroloji Dergisi*, 19(3), 181-191.

Čerňáková, M., Košťálová, D., 2002. Antimicrobial activity of berberine—A constituent ofMahonia aquifolium. *Folia Microbiologica*, 47(4), 375-378.

Chen, C., Lu, M., Pan, Q., Fichna, J., Zheng, L., Wang, K., Kreis, M., 2015a. Berberine improves intestinal motility and visceral pain in the mouse models mimicking diarrhea-predominant irritable bowel syndrome (IBS-D) symptoms in an opioid-receptor dependent manner. *PLoS One*, 10(12), e0145556.

Chen, C., Tao, C., Liu, Z., Lu, M., Pan, Q., 2015b. A randomized clinical trial of berberine hydrochloride in patients with diarrhea-predominant irritable bowel syndrome. *Phytotherapy Research*, 29(11), 1822-1827.

Chen, Y.X., Gao, Q.Y., Zou, T.H., Wang, B.M., Liu, S.D., Sheng, J.Q., Fang, J.Y., 2020. Berberine versus placebo for the prevention of recurrence of colorectal adenoma: a multicentre, double-blinded, randomised controlled study. *The Lancet Gastroenterology & Hepatology*, 5(3), 267-275.

Chey, W.D., Lembo, A.J., Lavins, B.J., Shiff, S.J., Kurtz, C.B., Currie, M.G., Johnston, J.M., 2012. Linaclotide for irritable bowel syndrome with constipation: a 26-week, randomized, double-blind, placebo-controlled trial to evaluate efficacy and safety. *Official Journal of the American College of Gastroenterology | ACG*, 107(11), 1702-1712.

Clavé, P., Acalovschi, M., Triantafillidis, J.K., Uspensky, Y.P., Kalayci, C., Shee, V., OBIS Study Investigators., 2011. Randomised clinical trial: otilonium bromide improves frequency of abdominal pain, severity of distention and time to relapse in patients with irritable bowel syndrome. *Alimentary Pharmacology & Therapeutics*, 34(4), 432-442.

Delzenne, N.M, Bindels, B., 2019. Food for thought about manipulating gut bacteria. *Nature*, 577(7788), 32-34.

Di Pierro, F., Bertuccioli, A., Giuberti, R., Saponara, M., Ivaldi, L., 2020. Role of a berberine-based nutritional supplement in reducing diarrhea in subjects with functional gastrointestinal disorders. *Minerva Gastroenterologica e Dietologica*, 66(1), 29-34.

Drossman, D.A., Chey, W.D., Johanson, J.F., Fass, R., Scott, C., Panas, R., Ueno, R., 2009. Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome–results of two randomized, placebo-controlled studies. *Alimentary Pharmacology & Therapeutics*, 29(3), 329-341.

Feng, A.W., Gao, W., Zhou, G.R., Yu, R., Li, N., Huang, X.L., Li, J.S., 2012. Berberine ameliorates COX-2 expression in rat small intestinal mucosa partially through PPARγ pathway during acute endotoxemia. *International Immunopharmacology*, 12(1), 182-188.

Feng, Y., Li, Y., Chen, C., Lin, X., Yang, Y., Cai, H., Jia, Y., 2013. Inhibiting roles of berberine in gut movement of rodents are related to activation of the endogenous opioid system. *Phytotherapy Research*, 27(10), 1564-1571.

Gu, L., Li, N., Li, Q., Zhang, Q., Wang, C., Zhu, W., Li, J. (2009). The effect of berberine *in vitro* on tight junctions in human Caco-2 intestinal epithelial cells. *Fitoterapia*, 80(4), 241-248.

Habtemariam, S., 2020. Berberine pharmacology and the gut microbiota: A hidden therapeutic link. *Pharmacological Research*, 155, 104722.

Hu, Y., Chen, X., Duan, H., Hu, Y., Mu, X., 2009. Chinese herbal medicinal ingredients inhibit secretion of IL-6, IL-8, E-selectin and TXB2 in LPS-induced rat intestinal microvascular endothelial cells. *Immunopharmacology and Immunotoxicology*, 31(4), 550-555.

Inadomi, J.M., Fennerty, M.B., Bjorkman, D., 2003. The economic impact of irritable bowel syndrome. *Alimentary Pharmacology & Therapeutics*, 18(7), 671-682.

Jeong, H.W., Hsu, K.C., Lee, J.W., Ham, M., Huh, J.Y., Shin, H.J., Kim, J.B., 2009. Berberine suppresses proinflammatory responses through AMPK activation in macrophages. *American Journal of Physiology-Endocrinology and Metabolism*, 296(4), E955-E964.

Jia, Q., Zhang, L., Zhang, J., Pei, F., Zhu, S., Sun, Q., Duan, L., 2019. Fecal microbiota of diarrhea-predominant irritable bowel syndrome patients causes hepatic inflammation of germ-free rats and berberine reverses it partially. *BioMed Research International*, 2019, 4530203.

Khan, S., Chang, L., 2010. Diagnosis and management of IBS. *Nature Reviews Gastroenterology & Hepatology*, 7(10), 565-581.

Khin, M.U., Myo, K., Nyunt, N.W., Aye, K., Tin, U., 1985. Clinical trial of berberine in acute watery diarrhoea. *British Medical Journal (Clinical Research Ed.)*, 291(6509), 1601-1605.

Kidd, P.M., 2009. Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. *Alternative Medicine Review*, 14(3), 226-246.

Kim, H.J., 2015. Berberine ameliorates allodynia induced by chronic constriction injury of the sciatic nerve in rats. *Journal of Medicinal Food*, 18(8), 909-915.

Kulkarni, S.K., Dhir, A., 2010. Berberine: a plant alkaloid with therapeutic potential for central nervous system disorders. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 24(3), 317-324.

Langmead, L., Chitnis, M., Rampton, D.S., 2002. Use of complementary therapies by patients with IBD may indicate psychosocial distress. *Inflammatory Bowel Diseases*, 8(3), 174-179.

Liang, Y., Xu, X., Yin, M., Zhang, Y., Huang, L., Chen, R., Ni, J., 2019. Effects of berberine on blood glucose in patients with type 2 diabetes mellitus: a systematic literature review and a meta-analysis. *Endocrine Journal*, 66(1), 51-63.

Longstreth, G.F., Thompson, W.G., Chey, W.D., Houghton, L.A., Mearin, F., Spiller, R.C., 2006. Functional bowel disorders. *Gastroenterology*, 130(5), 1480-1491.

Longstreth, G.F., Wilson, A., Knight, K., Wong, J., Chiou, C.F., Barghout, V., Ofman, J.J., 2003. Irritable bowel syndrome, health care use, and costs: a US managed care perspective. *The American Journal of Gastroenterology*, 98(3), 600-607.

Lovell, R.M., & Ford, A.C., 2012. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. *Clinical Gastroenterology and Hepatology*, 10(7), 712-721.

Mansueto, P., Seidita, A., D'Alcamo, A., Carroccio, A., 2015. Role of FODMAPs in patients with irritable bowel syndrome. *Nutrition in Clinical Practice*, 30(5), 665-682.

Ooi, S.L., Correa, D., Pak, S.C., 2019. Probiotics, prebiotics, and low FODMAP diet for irritable bowel syndrome–What is the current evidence?. *Complementary Therapies in Medicine*, 43, 73-80.

Palsson, O.S., Whitehead, W.E., 2013. Psychological treatments in functional gastrointestinal disorders: a primer for the gastroenterologist. *Clinical Gastroenterology and Hepatology*, 11(3), 208-216.

Payne, A., Blanchard, E.B., 1995. A controlled comparison of cognitive therapy and self-help support groups in the treatment of irritable bowel syndrome. *Journal of Consulting and Clinical Psychology*, 63(5), 779-786.

Rabbani, G.H., Butler, T., Knight, J., Sanyal, S.C., Alam, K., 1987. Randomized controlled trial of berberine sulfate therapy for diarrhea due to enterotoxigenic *Escherichia coli* and *Vibrio cholerae*. *Journal of Infectious Diseases*, 155(5), 979-984.

Round, J.L., Mazmanian, S.K., 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, 9(5), 313-323.

Saha, P., Bhattacharjee, S., Sarkar, A., Manna, A., Majumder, S., Chatterjee, M., 2011. Berberine chloride mediates its anti-leishmanial activity via differential regulation of the mitogen activated protein kinase pathway in macrophages. *PLoS One*, 6(4), e18467.

Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition*, 130(8), 2073S-2085S.

Schmulson, M.J., Drossman, D.A., 2017. What is new in Rome IV. *Journal of Neurogastroenterology and Motility*, 23(2), 151-163.

Soares, R.L., 2014. Irritable bowel syndrome: a clinical review. *World Journal of Gastroenterology: WJG*, 20(34), 12144-12160.

Sun, D., Abraham, S.N., Beachey, E.H., 1988. Influence of berberine sulfate on synthesis and expression of Pap fimbrial adhesin in uropathogenic *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*, 32(8), 1274-1277.

Sun, S., Wang, K., Lei, H., Li, L., Tu, M., Zeng, S., Jiang, H., 2014. Inhibition of organic cation transporter 2 and 3 may be involved in the mechanism of the antidepressant-like action of berberine. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 49, 1-6.

Talley, N.J., Spiller, R., 2002. Irritable bowel syndrome: a little understood organic bowel disease?. *The Lancet*, 360(9332), 555-564.

Tang, Q.L., Lai, M.L., Zhong, Y.F., Wang, A.M., Su, J.K., Zhang, M.Q., 2013. Antinociceptive effect of berberine on visceral hypersensitivity in rats. *World Journal of Gastroenterology: WJG*, 19(28), 4582-4589.

Taylor, C.T., Winter, D.C., Skelly, M.M., O'Donoghue, D.P., O'Sullivan, G.C., Harvey, B.J., Baird, A.W., 1999. Berberine inhibits ion transport in human colonic epithelia. *European Journal of Pharmacology*, 368(1), 111-118.

Wang, Z.C., Wang, J., Chen, H., Tang, J., Bian, A.W., Liu, T., Yang, F., 2020. Synthesis and anticancer activity of novel 9, 13-disubstituted berberine derivatives. *Bioorganic & Medicinal Chemistry Letters*, 30(2), 126821.

Webb, A.N., Kukuruzovic, R., Catto-Smith, A.G., Sawyer, S.M., 2007. Hypnotherapy for treatment of irritable bowel syndrome. *Cochrane Database of Systematic Reviews*, (4), CD005110.

Wu, D.Z., Yuan, J.Y., Shi, H.L., Hu, Z.B., 2008. Palmatine, a protoberberine alkaloid, inhibits both Ca^{2+}-and cAMP-activated Cl^- secretion in isolated rat distal colon. *British Journal of Pharmacology*, 153(6), 1203-1213.

Yan, F., Wang, L., Shi, Y., Cao, H., Liu, L., Washington, M.K., Polk, D.B., 2012. Berberine promotes recovery of colitis and inhibits inflammatory responses in colonic

macrophages and epithelial cells in DSS-treated mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 302(5), G504-G514.

Yin, J., Xing, H., Ye, J., 2008. Efficacy of berberine in patients with type 2 diabetes mellitus. *Metabolism*, 57(5), 712-717.

Yu, F., Ao, M., Zheng, X., Li, N., Xia, J., Li, Y., Chen, X.D., 2017. PEG–lipid–PLGA hybrid nanoparticles loaded with berberine–phospholipid complex to facilitate the oral delivery efficiency. *Drug Delivery*, 24(1), 825-833.

Yu, Z.C., Cen, Y.X., Wu, B.H., Wei, C., Xiong, F., Li, D.F., Yao, J., 2019. Berberine prevents stress-induced gut inflammation and visceral hypersensitivity and reduces intestinal motility in rats. *World Journal of Gastroenterology*, 25(29), 3956-3971.

Zhang, L.S., Zhang, J.H., Feng, R., Jin, X.Y., Yang, F.W., Ji, Z.C., Li, X.M., 2019. Efficacy and safety of berberine alone or combined with statins for the treatment of hyperlipidemia: a systematic review and meta-analysis of randomized controlled clinical trials. *The American Journal of Chinese Medicine*, 47(04), 751-767.

Zhang, M., Long, Y., Sun, Y., Wang, Y., Li, Q., Wu, H., Mei, Q., 2011. Evidence for the complementary and synergistic effects of the three-alkaloid combination regimen containing berberine, hypaconitine and skimmianine on the ulcerative colitis rats induced by trinitrobenzene-sulfonic acid. *European Journal of Pharmacology*, 651(1-3), 187-196.

Zhang, Y., Li, X., Zou, D., Liu, W., Yang, J., Zhu, N., Ning, G., 2008. Treatment of type 2 diabetes and dyslipidemia with the natural plant alkaloid berberine. *The Journal of Clinical Endocrinology & Metabolism*, 93(7), 2559-2565.

Zhou, H., Mineshita, S., 2000. The effect of berberine chloride on experimental colitis in rats *in vivo* and *in vitro*. *Journal of Pharmacology and Experimental Therapeutics*, 294(3), 822-829.

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RESEARCH ARTICLE

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Pergularia daemia (Apocynaceae) mitigates rifampicin-induced hepato-renal injury: potentials in the management of liver and kidney diseases

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ABSTRACT

Medicinal potentials of *Pergularia daemia* leaves in managing hepato-renal toxicity induced by rifampicin were investigated. Twenty-five (25) Wistar rats were randomly placed into five groups containing five animals each. All the animals, except group I, were orally exposed to 250 mg/kg bwt rifampicin and administered different treatments. Specific liver and kidney biomarkers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined. In addition, malondialdehyde (MDA), lipid profile, superoxide dismutase (SOD), catalase (CAT), as well as reduced glutathione (GSH) were determined in the serum, liver, and kidney homogenates of experimental animals. Results indicate that exposure to rifampicin caused significant depletion in SOD and CAT relative to the control animals. Lipid profile was deranged, while ALT, AST, ALP, urea, uric acid, bilirubin, creatine kinase, and MDA level were elevated by rifampicin exposure. All deranged biochemical indices, as well as distorted histoarchitecture, were restored dose-dependently after treatment with *P. daemia*. In conclusion, *P. daemia* ameliorated rifampicin toxicity on the liver and kidney as indicated in the restoration of all deranged biochemical and histopathological indices measured. Hence, it is a potential therapeutic agent that can be harnessed as panacea to the menace of liver and kidney diseases.

1. Introduction

Plants are enriched with phytochemicals that offer protection from stressors, thereby ensuring their survival within the ecosystem. These phytochemicals form the bulk of potential raw materials for rational drug synthesis in modern medicine (Muller et al., 2006). Over eighty percent of plants so far identified are rich in phytochemicals with therapeutic potentials to treat life-threatening diseases in humans. Unfortunately, the medicinal potential of these plants have not been fully exploited in treating human diseases (Krishnaiah et al., 2011). Hence, a deliberate research effort to investigate these plants for their potential benefits has been on top gear in recent times. The output of such research efforts could proffer a cheap therapeutic alternative for managing several pathological conditions, thereby strengthening the healthcare system (Jelkmann, 2001).

Pergularia daemia (Apocynaceae) is a plant popularly recognized by African traditional medical practitioners for its importance in treating several ailments (Chandak and Dighe, 2019). In folkloric medicine, *P. daemia* is useful in treating jaundice, aiding digestion, as an expectorant, analgesic, anticonvulsant, antiasthma, and antipyretic. Its root extract has been used to treat leprosy, mental derangement, anemia, and diarrhea (Chandak and Dighe, 2019).

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Oral administration of its leaf extract regulates menstruation and uterine function (Dosumu et al., 2019). In addition, reports suggest its potential antidiabetic, anticatarrhal, and antibronchitis properties (Chandak and Dighe, 2019). Its leaves contain active principles such as 4-4chlorobenzoyl-1-cyclohexoyl-5-tosylamino-1,H-123 zole, methyl ester pentadecoinic acid, 14-methyl-methyl ester ethyl 9-12-15-octodecotrionate, hexadecanoic acid, flavonoids, tannins, alkaloids, phenols and steroids (Chandak, 2010; Nithyatharani and Kavitha, 2018). Perhaps, these phytoconstituents are responsible for their medicinal relevance.

A bactericidal antibiotic drug, rifampicin is a semisynthetic component derived from streptomyces species. It is usually used as a first-line drug for treating tuberculosis globally (Eminzade et al., 2008). Drug-induced hepatotoxicity and nephrotoxicity are gradually assuming a global dimension in recent times. Specifically, reports have suggested that drug-induced hepatotoxicity is the leading cause of drug withdrawal or non-approval by the Food and Drug Administration (FDA) in the United States (US) (Kohli et al., 2000; Panich et al., 2012). Over a thousand drugs have predisposed their users to liver and kidney damage (Larrey, 2000; Biour et al., 2004; Ueno et al., 2002; Antonyuk et al., 2009). In all acute liver and kidney failures, 50% are caused by drugs administered in their management (Panich et al., 2012). Apart from its primary role as anti-tuberculosis, the metabolite of rifampicin metabolism has the potential to induce oxidative assault on the liver and kidney. Evidence suggests that rifampicin acts by causing derangement in heme biosynthesis, leading to the accumulation of hepatotoxic protoporphyrin (Yue-Ming et al., 2014).

Globally, the prevalence of liver and kidney diseases is a worrisome dimension of public health concern. Hence, a cheap but efficient therapeutic alternative must be sought to avoid further escalation. Considering the numerous medicinal relevance of *P. daemia* in folkloric medicine, it is necessary to investigate its hepatoprotective and nephroprotective potentials. This is the reason for the study.

2. Materials and methods

2.1. Plant materials

P. daemia leaves were harvested from a farm within Ekiti State University, Campus, Iworoko Road, Ado Ekiti, in May 2021 and botanically identified at the Department of Plant Science, Ekiti State University, Ado Ekiti. Harvested leaves were rinsed with water, air-dried in the laboratory, pulverized, and then stored in an airtight container.

2.2. Reagents and chemicals

All reagents and chemicals were of high analytical grade. All diagnostic kits used were products of Randox Chemical Ltd. England.

2.3. Extraction of the plant material

P. daemia leaves were air-dried for 24 days and pulverized. One hundred and seventy-three (173) g of the powdered leaves were extracted in 1000 ml of distilled water for 72 hours. It was then filtered using cheesecloth and freeze-dried to obtain the dried extract. The extract was kept in a closed container and refrigerated at 4 °C for further studies.

2.4. Animals protocol

Twenty-five (25) Wistar albino rats weighing 150 – 170 g were obtained from The Animal House, Department of Science Technology, The Federal Polytechnic, Ado Ekiti and housed in clean wire meshed cages under standard conditions temperature (24 ± 1 °C), relative humidity, and 12/12-hour light and dark cycle. They were given unrestricted access to food (commercial palletized diet from Vital Feed Mill) and drinking water ad libitum. Beddings for experimental rats were routinely changed and replaced every day throughout the experimental period (Table 1).

Table 1. Animal treatment/experimental design

Groups	Treatment
I	Distilled water only
II	Rifampicin (250 mg/kg bwt) only
III	Rifampicin (250 mg/kg bwt) + *P. daemia* (50 mg/kg bwt)
IV	Rifampicin (250 mg/kg bwt) + *P. daemia* (100 mg/kg bwt)
V	Rifampicin (250 mg/kg body weight) + silymarin (100 mg/kg bwt

bwt: body weight

2.5. Dissection of rats

Experimental rats were decapitated and dissected after very mild ether anesthesia to obtain the liver and kidney. Blood was collected by cardiac puncture into EDTA sample bottles and allowed to stand for 1 hour. Serum was obtained by centrifugation at 3000 rpm for 15 min at 25 °C. The clear supernatant (serum) was collected and used to estimate serum biochemical parameters.

2.6. Preparation of homogenates

Liver and kidney tissues were trimmed of fat, washed in distilled water, blotted with filter paper, and weighed. The kidney and liver were homogenized separately in ten volumes of the homogenizing phosphate buffer (pH 7.4) using a Teflon homogenizer. The resulting homogenates were centrifuged separately at 3000 rpm at 4 °C for 30 min. The supernatant obtained was stored in a refrigerator for further biochemical analyses.

2.7. Serum enzyme biomarkers

2.7.1. Creatine kinase (Ck-Mb) activity

The level of creatine kinase was determined according to Vanderlinde (1981). One milliliter (1 ml) each of imidazole buffer (10 mM, pH 6.6), creatine phosphate (30 mM), glucose (20 mM), N-acetyl-cysteine (20 mM), magnesium acetate (10 mM), ethylenediaminetetraacetic acid (2 mM), ADP (2 mM), NADP (2 mM), AMP (5 mM), DAPP (10 µM), G6PDH (≥ 2.0 ku/l) and HK(≥ 2.15 ku/l) was pipetted into a thermostatic cuvette. The mixture was then incubated at 37 °C, after which 50 µl of serum was added. The reaction components were thoroughly mixed, and absorbance was read immediately for 5 min at 30-sec intervals at a wavelength of 340 nm. Change in absorbance per minute was calculated (ΔAbs/min).

2.7.2. Assay of aspartate aminotransferase (AST)

The activity of AST in the serum and organs homogenates was determined as described by Reitman and Frankel (1957). One hundred microliters of serum and organ homogenates were mixed separately with phosphate buffer (100 mmol/l, pH 7.4), L-aspartate (100 mmol/l), and α-oxoglutarate (2 mmol/l). The mixture was incubated for exactly 30 min at 37 °C. 0.5 ml of 2,4-dinitrophenyl

hydrazine (2 mmol/l) was added to the reaction mixture and allowed to stand for 20min at 25 °C. After that, 5.0 ml of NaOH (0.4 mol/l) was added, and absorbance was read at 546 nm against the reagent blank after 5 min.

2.7.3. Assay of alanine aminotransferase (ALT) activity

The principle described by Reitman and Frankel (1957) was followed in the assay of ALT using a commercially available assay kit (Randox Laboratories, UK) according to the manufacturer's instructions. Reagent 1 (0.5 ml) containing phosphate buffer (100 mmol/l, pH 7.4), L-alanine (200 mmol/l), and α-oxoglutarate (2.0 mol/l) was added to 0.1 ml of serum and organs homogenates in a separate test tube, and the mixture was incubated at 37 °C for 30 min. Exactly 0.5 ml of reagent 2 (R2) containing 2, 4-dinitrophenylhydrazine (2.0 mmol/l) was added, and the solution and re-incubated at 20 °C for 20 min. Finally, 5 ml of NaOH was added, and the solution was allowed to stand for 5 min at 25 °C, and its absorbance was read at 546 nm.

2.7.4. Assay of alkaline phosphatase (ALP) activity

Assay of serum ALP was performed by the method of Englehardt (1970) using commercial assay kits (Randox Laboratories, UK) according to the instructions of the manufacturer. Exactly 1.0 ml of the reagent (1 mol/l diethanolamine buffer pH 9.8, 0.5 mmol/l $MgCl_2$; substrate: 10 mmol/l *p*-nitrophenol phosphate) was added to 0.02 ml of the serum sample and mixed. Absorbance was taken at 405 nm for 3 min at 1 min intervals.

2.8. Serum lipid profile analysis

2.8.1. Estimation of total cholesterol

Total cholesterol level was determined based on the method of Trinder (1969) using commercially available kits (Randox Laboratories, UK). Ten microliters (10 µl) of standard and 10 µl serum samples were pipetted into labeled test tubes. One milliliter (1 ml) of working reagent containing; pipes buffer (80 mmol/l at pH 6.8), 4-amino antipyrine (0.25 mmol/l), phenol (6 mmol/l), peroxidase (≥ 0.5 U/ml), cholesterol esterase ion (≥ 0.15 U/ml) and cholesterol oxidase (0.10 U/ml) was added into all the tubes. The reaction mixtures were mixed thoroughly and incubated for 10 minutes. The absorbance of the sample (A_{sample}) was read at 500 nm against the reagent blank.

2.8.2. Evaluation of concentration of triglyceride

Triglycerides level in the serum and organs' homogenates was determined based on Tietz's (1995) method using commercially available kits (Randox Laboratories, UK). Triglyceride standard (10 µl) and serum (10 µl) were pipetted into labeled test tubes. One milliliter of the working reagents; R1a (buffer) containing pipes buffer (40mmol/l, pH 7.6), 4-chloro-phenol (5.5 mmol/l), magnesium-ion (17.5 mmol/l); R1b [(enzyme reagent containing 4-amino phenazone (0.5 mmol/l), ATP (1.0 mmol/l), lipase (≥ 150 U/ml), glycerol-kinase (≥ 0.4 U/ml), glycerol-3-phosphate oxidase (≥ 1.5 U/ml) and peroxidase (≥ 0.5 U/ml)] was added into all the tubes. The reaction components were thoroughly mixed and incubated for 10 min at room temperature. Absorbance was read at 546 nm against the blank.

2.8.3. High-density lipoprotein (HDL-c)-cholesterol assay

The method of Grove (1979) was adopted in estimating HDL-cholesterol in the serum. The precipitation reaction mixture contained 200 µl of the serum, 200 µl of the cholesterol standard, and 500 µl of the diluted precipitant R1 (0.55 mM phosphotungstic acid, 25 mM magnesium chloride) was mixed and allowed to stand for 10 min at room temperature. It was then centrifuged for 10 min at 4000 rpm to obtain a clear supernatant. The mixture was decanted within 2 h to determine the cholesterol content by the CHOD-PAP reaction method.

2.8.3.1. Cholesterol CHOD-PAP assay

One hundred microliters (100 µl) of the sample supernatant was added to 1 ml of cholesterol reagent and mixed in a test tube. The standard test tube contained 100 µl of the cholesterol standard supernatant and 1 ml of cholesterol reagent. The reagent mixture was mixed thoroughly and incubated for 10 min at 25 °C. The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) was then measured at 500 nm against the reagent blank within 1 h.

2.8.4. Low-density lipoprotein (LDL) - cholesterol

The concentration of low-density lipoprotein in the serum was calculated using the formula of Friedewald et al. (1972) as given below:

LDL cholesterol = Total cholesterol – Triglycerides/5 – HDL-cholesterol

2.9. Antioxidant assay

2.9.1. Determination of catalase activity

Catalase activity was determined using the method described by Sinha (1972). Two hundred microliter each of the serum, liver, and kidney homogenates was mixed separately with 0.8 ml distilled water to give 1 in 5 dilutions of the sample. The assay mixture contained 2 ml of hydrogen peroxide solution (800 µmol) and 2.5 ml of phosphate buffer in a 10 ml flat bottom flask. Properly diluted enzyme preparation (0.5 ml) was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1 ml portion of the reaction mixture was withdrawn and blown into a 1 ml dichromate/acetic acid reagent at 60 s intervals. The hydrogen peroxide content of the withdrawn sample was determined by the method described below.

Catalase activity =

H_2O_2 consumed = 800 – Concentration of H_2O_2 remaining

The concentration of H_2O_2 remaining was extrapolated from the standard curve for catalase activity

2.9.2. Determination of superoxide dismutase (SOD)

Superoxide dismutase activity was determined by the method of Misra and Fridovich (1972). One milliliter of serum, liver, and kidney homogenates was diluted separately in 9 ml of distilled water to make a 1 in 10 dilutions. An aliquot of the diluted sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. The reaction was initiated by adding 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture, which was quickly mixed by inversion. The reference cuvette contained 2.5 ml

buffer, 0.3 ml of the substrate (adrenaline), and 0.2 ml of water. An increase in absorbance at 480 nm was monitored every 30 s for 150 s.

2.9.3. Determination of reduced glutathione (GSH) level

The amount of GSH was determined using the method of Beutler et al. (1963). Exactly 0.2 ml of serum, liver, and kidney was added to 1.8 ml of distilled water, followed by the addition of 3 ml of precipitating solution. The resulting solution was then mixed thoroughly, allowed to stand for 5 minutes, and filtered. One milliliter (1 ml) of the filtrate was added to 4 ml of 0.1 M phosphate buffer pH 7.4. Finally, 0.5 ml of Ellman reagent was added. A blank was prepared with 4 ml of the 0.1 M phosphate buffer, 1 ml of diluted precipitating solution (3 parts to 2 parts of distilled water), and 0.5 ml of the Ellman reagent. The absorbance of the resulting mixture was measured at 412 nm against a reagent blank.

2.9.4. Determination of total protein (TP) in serum

The amount of protein in the serum, liver, and kidney homogenates was measured according to the method of Weichselbaum (1946) using commercially available kits (Randox Laboratories, UK). One milliliter (1 ml) of Reagent R1 containing sodium hydroxide (100 mmol/l), Na-K-tartrate (18 mmol/l), potassium iodide (15 mmol/l), and cupric sulfate (6 mmol/l) was added to 0.02 ml of the serum sample. The mixture was incubated at 25 °C, and its absorbance was measured against the reagent blank at a wavelength of 546 nm.

Table 2a. Effects of aqueous extract of *P. daemia* on selected biomarkers of liver function in rifampicin-exposed rats

Parameter	I	II	III	IV	V
ALT (U/l)	40.71 ± 1.23^a	77.23 ± 1.42^b	69.23 ± 1.07^b	57.08 ± 1.00^a	51.33 ± 1.27^a
AST (U/l)	54.33 ± 1.71^a	96.14 ± 2.41^b	78.33 ± 1.88^a	62.54 ± 2.02^a	57.23 ± 1.72^a
ALP (U/l)	47.25 ± 0.72^a	73.84 ± 0.81^b	71.23 ± 0.61^a	57.07 ± 0.53^a	46.88 ± 1.39^a
T. BIL (mg/dl)	31.18 ± 0.67^a	56.18 ± 0.54^b	47.32 ± 0.30^a	40.20 ± 0.60^a	31.72 ± 3.82^a

Table 2b. Effects of aqueous extract of *P. daemia* on selected biomarkers of kidney function in rifampicin-exposed rats

Parameter	I	II	III	IV	V
ALT (U/l)	17.27 ± 2.13^a	40.29 ± 1.77^b	33.44 ± 1.40^b	26.42 ± 0.89^a	23.63 ± 1.03^a
AST (U/l)	21.60 ± 1.04^a	56.82 ± 1.73^b	39.22 ± 1.06^a	22.50 ± 1.31^a	25.06 ± 1.25^a
ALP (U/l)	31.21 ± 1.65^a	53.66 ± 1.98^b	41.86 ± 0.75^a	32.86 ± 0.65^a	29.32 ± 0.90^a
Urea (mg/dl)	47.82 ± 0.67^a	70.06 ± 0.83^b	62.08 ± 0.43^a	50.20 ± 0.52^a	53.76 ± 1.61^a
U. acid (mg/dl)	29.54 ± 0.26^a	54.23 ± 0.78^b	39.57 ± 0.55^a	28.32 ± 0.41^a	32.89 ± 1.62^a
C. K. (U/l)	27.61 ± 0.83^a	49.17 ± 0.94^b	36.42 ± 0.85^a	27.00 ± 0.53^a	25.73 ± 0.85^a

Table 2c. Effects of aqueous extract of *P. daemia* on selected serum biomarkers of liver and kidney function in rifampicin-exposed rats

Parameter	I	II	III	IV	V
ALT (U/l)	56.17 ± 3.04^a	88.45 ± 2.43^b	60.23 ± 2.63^a	55.13 ± 2.09^a	59.33 ± 1.34^a
AST (U/l)	71.26 ± 1.71^a	127.13 ± 2.60^b	96.51 ± 1.54^a	70.48 ± 1.60^a	75.22 ± 1.56^a
ALP (U/l)	62.13 ± 1.32^a	108.52 ± 1.32^b	75.39 ± 1.31^a	68.18 ± 1.53^a	67.11 ± 2.09^a
Urea (mg/dl)	38.77 ± 0.66^a	64.39 ± 0.62^b	54.20 ± 0.70^a	37.21 ± 0.65^a	45.26 ± 0.76^a
U. acid (mg/dl)	19.17 ± 0.43^a	35.43 ± 0.52^b	31.02 ± 0.34^a	24.88 ± 0.41^a	21.17 ± 0.68^a
C.K. (U/l)	20.33 ± 0.37^a	44.58 ± 0.44^b	33.41 ± 1.01^a	25.11 ± 0.58^a	23.66 ± 0.22^a
T.BIL (mg/dl)	42.25 ± 0.64^a	69.30 ± 1.02^b	50.26 ± 0.55^a	41.31 ± 0.86^a	45.37 ± 1.84^a

Data represent the mean ± SEM of the experiment performed in triplicate. I: Administered water only, II: Rats exposed to rifampicin (250 mg/kg bwt) only, III: Rats treated with *P. daemia* at 50 mg/kg bwt after exposure to rifampicin, IV: Rats treated with *P. daemia* at 100 mg/kg bwt after exposure to rifampicin, V: Rats treated with silymarin at 100 mg/kg bwt after exposure. 'b' represents a significant difference from the control 'a' at $p = 0.05$.

2.10. Statistical analysis

Data were expressed as mean ± SEM. Statistical evaluation was done using One Way Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) using SPSS 11.09 for Windows. The significance level was set at $p = 0.05$

3. Results and discussion

Exposure of experimental rats to rifampicin at 250 mg/kg bwt caused a significant increase in hepatic and renal alanine aminotransferase, aspartate aminotransferase, and alkaline phosphate. Treatment of exposed rats with *P. daemia* leaf extract restored these biomarkers to the basal dose-dependent manner similar to animals treated with silymarin. The same trend was observed for urea, uric acid, total bilirubin, and creatine kinase in the serum, liver, and kidney homogenates (Table 2a-c). Similarly, oral administration of rifampicin at 250 mg/kg bwt resulted in marked derangement in lipid profile relative to the control. This caused a surge in cholesterol, triglyceride, and low-density lipoprotein, as well as marked depletion in high-density lipoprotein (HDL) in the liver, kidney, and serum (Table 3a-c). Treatment with *P. daemia* dose-dependently restored the deranged lipid profile to a level comparable with animals not exposed to rifampicin. Moreover, catalase and superoxide dismutase activities in the serum, kidney, and liver were significantly inhibited following exposure to rifampicin. Reduced glutathione was markedly diminished in the serum, and organs homogenated after exposure to rifampicin. However, treatment with *P. daemia* relieved the inhibition imposed on the enzymes and restored GSH to a level comparable to the control animals (Table 4a-c). Finally, there was an observed sharp increase in MDA levels in the liver and kidney of experimental animals following rifampicin exposure (Figure 1). This was brought back to normal dose-dependent when intoxicated animals were treated with *P. daemia*.

In recent times, the prevalence of kidney and liver diseases poses a serious threat to public health and must be addressed urgently. Developing nations, in particular, should pay attention to finding a cheap, locally available, and potent therapeutic alternative in the management of these diseases. Up to 40% of liver and kidney failure are traceable to side effects of conventional drugs administered in

the management of ailments (Kosanam and Boyina, 2015). Specifically, hepato-renal injuries have been linked to oxidative assaults caused by specific bioactivated intermediates produced in biotransformation (Sharma and Sharma, 2015; Basheer et al., 2017). The use of plants for medication is an age-long practice that has proven effective (Sentman et al., 2006; Abirami et al., 2014).

In the present study, there was a significant increase in AST, ALT, and ALP levels in the serum and organs' homogenates following exposure to rifampicin (Table 2a-c). Obviously, this is due to free radicals-induced leakage of these enzymes from their initial cellular compartments into the bloodstream. The leakage must have resulted from a compromise in membrane integrity via lipid peroxidation, as Rana et al. (2006) and Kim et al. (2017) suggested. Treatment with *P. daemia* extract reversed the surge in the level of these biomarker enzymes, perhaps via inhibition of lipid peroxidation. As a result, membrane integrity must have been restored, thereby blocking further leakage of these enzymes into the bloodstream. This healing ability of *P. daemia* extract can be attributed to its antioxidant potential, as earlier reported (Vaithiyanathan and Mirunalini, 2016).

Table 3a. Effects of aqueous extract of *P. daemia* on hepatic lipid profile of rifampicin-exposed rats

Parameter	I	II	III	IV	V
CHOL (mg/dl)	56.08 ± 1.86^a	97.23 ± 1.42^b	71.13 ± 1.01^a	64.43 ± 1.08^a	52.33 ± 1.34^a
TRIG (mg/dl)	41.33 ± 0.76^a	86.50 ± 0.42^b	69.26 ± 0.59^a	52.09 ± 0.94^a	39.43 ± 1.13^a
HDL (mg/dl)	24.72 ± 0.22^a	18.26 ± 0.24^b	18.76 ± 0.51^a	21.33 ± 0.33^a	26.31 ± 0.59^a
LDL (mg/dl)	33.76 ± 0.87^a	57.81 ± 0.11^b	51.25 ± 0.06^a	40.42 ± 0.15^a	33.04 ± 0.62^a

Table 3b. Effects of aqueous extract of *P. daemia* on renal lipid profile of rifampicin-exposed rats

Parameter	I	II	III	IV	V
CHOL (mg/dl)	30.07 ± 1.05^a	53.24 ± 1.03^b	47.23 ± 1.07^b	36.27 ± 0.76^a	38.63 ± 2.23^a
TRIG (mg/dl)	11.82 ± 0.14^a	26.48 ± 0.33^b	23.08 ± 0.49^b	17.80 ± 0.24^a	13.43 ± 0.29^a
HDL (mg/dl)	8.26 ± 0.95^a	6.08 ± 0.63^b	6.45 ± 0.56^a	7.03 ± 0.34^a	8.42 ± 0.68^a
LDL (mg/dl)	13.26 ± 0.77^a	31.27 ± 0.57^b	26.80 ± 0.38^b	16.29 ± 0.40^a	12.13 ± 0.48^a

Table 3c. Effects of aqueous extract of *P. daemia* on serum lipid profile of rifampicin-exposed rats

Parameter	I	II	III	IV	V
CHOL (mg/dl)	52.16 ± 1.14^a	87.46 ± 1.38^b	73.23 ± 1.05^a	66.20 ± 1.73^a	58.27 ± 1.56^a
TRIG (mg/dl)	37.51 ± 1.61^a	70.39 ± 1.45^b	63.71 ± 0.55^a	48.08 ± 1.09^a	40.14 ± 1.72^a
HDL (mg/dl)	9.42 ± 0.02^a	5.10 ± 0.07^b	5.63 ± 0.05^a	6.47 ± 0.01^a	8.79 ± 0.29^a
LDL (mg/dl)	22.36 ± 0.72^a	39.53 ± 0.68^b	34.48 ± 0.72^b	29.22 ± 0.63^a	24.67 ± 0.72^a

Data represent the mean ± SEM of the experiment performed in triplicate. I: Administered water only, II: Rats exposed to rifampicin (250 mg/kg bwt) only, III: Rats treated with *P. daemia* at 50 mg/kg bwt after exposure to rifampicin, IV: Rats treated with *P. daemia* at 100 mg/kg bw after exposure to rifampicin, V: Rats treated with silymarin at 100 mg/kg bwt after exposure. 'b' represents a significant difference from the control 'a' at $p = 0.05$.

Table 4a. Effects of aqueous extract of *P. daemia* on selected enzymic and non-enzymic antioxidant parameters in the liver of rifampicin-exposed rats

Parameter	I	II	III	IV	V
SOD (U/mg protein)	6.14 ± 0.14^a	2.36 ± 0.19^b	3.81 ± 0.12^a	5.92 ± 0.10^a	6.20 ± 1.23^a
CAT (µmol/min)	4.70 ± 0.13^a	1.63 ± 0.16^b	2.74 ± 0.22^a	3.82 ± 0.14^a	4.45 ± 1.20^a
GSH (mmol)	5.77 ± 0.03^a	2.93 ± 0.08^b	3.80 ± 0.05^a	4.63 ± 0.10^a	5.43 ± 0.08^a
T. P (mg/ml)	2.64 ± 0.13^a	1.04 ± 0.09^b	1.93 ± 0.10^a	2.45 ± 0.13^a	2.33 ± 0.78^a

Table 4b. Effects of aqueous extract of *P. daemia* on selected enzymic and non-enzymic antioxidant parameters in the kidney of rifampicin-exposed rats

Parameter	I	II	III	IV	V
SOD (U/mg protein)	3.48 ± 0.27^a	1.93 ± 0.19^b	2.63 ± 0.11^a	3.15 ± 0.13^a	3.15 ± 0.53^a
CAT (µmol/min)	2.77 ± 0.10^a	1.88 ± 0.26^b	2.04 ± 0.10^a	2.82 ± 0.16^a	2.58 ± 0.20^a
GSH (mmol)	3.23 ± 0.03^a	3.04 ± 0.02^b	3.17 ± 0.07^a	3.30 ± 0.04^a	3.19 ± 0.12^a
T. P (mg/ml)	1.73 ± 0.01^a	1.06 ± 0.01^b	1.24 ± 0.05^a	1.56 ± 0.07^a	1.44 ± 0.07^a

Table 4c. Effects of aqueous extract of *P. daemia* on selected enzymic and non-enzymic antioxidant parameters in the serum of rifampicin-exposed rats

Parameter	I	II	III	IV	V
SOD (U/mg protein)	4.29 ± 0.09^a	2.42 ± 0.07^b	3.61 ± 0.03^a	4.09 ± 0.07^a	4.34 ± 0.21^a
CAT (µmol/min)	3.06 ± 0.12^a	1.76 ± 0.14^b	2.39 ± 0.08^a	3.12 ± 0.10^a	2.93 ± 0.20^a
GSH (mmol)	2.66 ± 0.01^a	2.08 ± 0.02^b	2.26 ± 0.06^a	2.47 ± 0.07^a	2.49 ± 0.98^a
T. P (mg/ml)	2.08 ± 0.20^a	1.02 ± 0.18^b	1.39 ± 0.13^a	1.87 ± 0.14^a	2.17 ± 0.08^a

Data represent the mean ± SEM of the experiment performed in triplicate. I: Administered water only, II: Rats exposed to rifampicin (250 mg/kg bwt) only, III: Rats treated with *P. daemia* at 50 mg/kg bwt after exposure to rifampicin, IV: Rats treated with *P. daemia* at 100 mg/kg bwt after exposure to rifampicin, V: Rats treated with silymarin at 100 mg/kg bwt after exposure. 'b' represents a significant difference from the control 'a' at $p = 0.05$.

Serum bilirubin level was significantly elevated in the present study following exposure of experimental animals to rifampicin (Table 2a-c). It has been reported that rifampicin hinders bilirubin uptake, resulting in a subclinical level of unconjugated hyperbilirubinemia. Similarly, a blockage of the exporter pump specific for exporting bile could result in conjugated hyperbilirubinemia (Saukkonen et al.,

2006; Byrne et al. ,2002). Other reports also indicate that deficient bilirubin clearance or inefficient secretion at the canalicular level may trigger hyperbilirubinemia (Grosset and Leventis, 1983; Capelle et al., 1972). Treatment with *P. daemia* extract restored the bilirubin level to that comparable with animals not exposed at all. This is due to the antioxidant phytochemicals present in the extract, as earlier reported (Maheshwari and Vijayarengan, 2021).

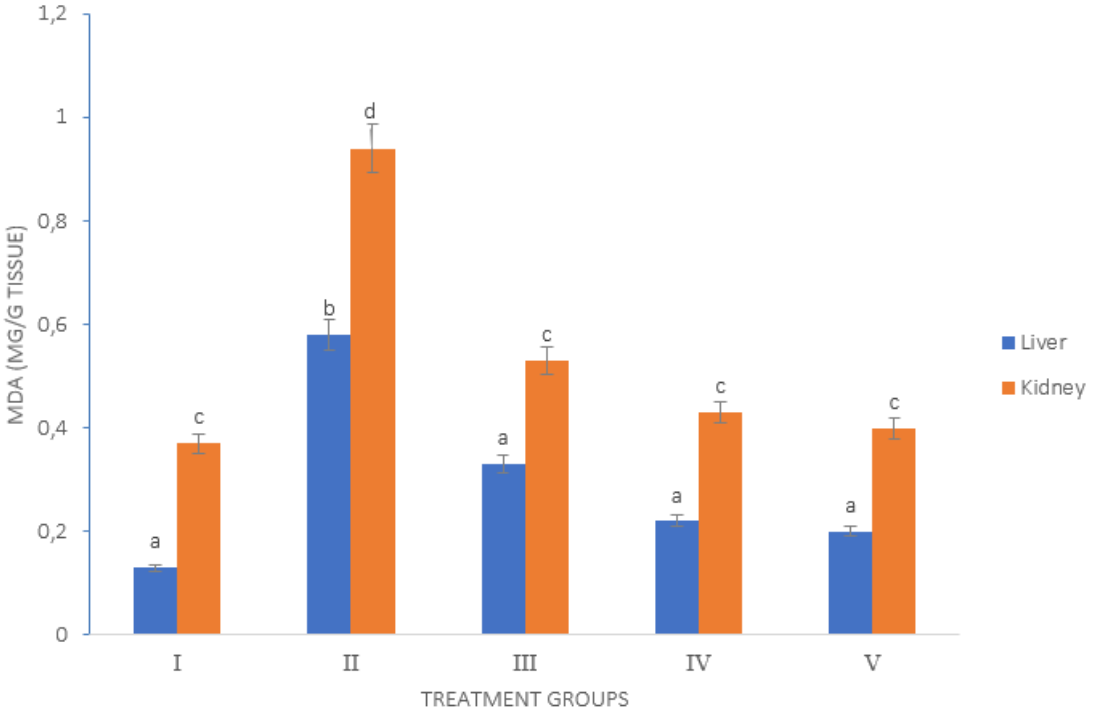

Figure 1. Effect of *P. daemia* on lipid peroxidation in the liver and kidney of rifampicin-exposed rats

Data represent the mean ± SEM of the experiment performed in triplicate. I: Administered water only, II: Rats exposed to rifampicin (250 mg/kg bw) only, III: Rats treated with *P. daemia* at 50 mg/kg bw after exposure to rifampicin, IV: Rats treated with *P. daemia* at 100 mg/kg bw after exposure to rifampicin, V: Rats treated with silymarin at 100 mg/kg bw after exposure. 'c' and 'd' represent a significant difference from the control 'a' and 'b', respectively at $p = 0.05$.

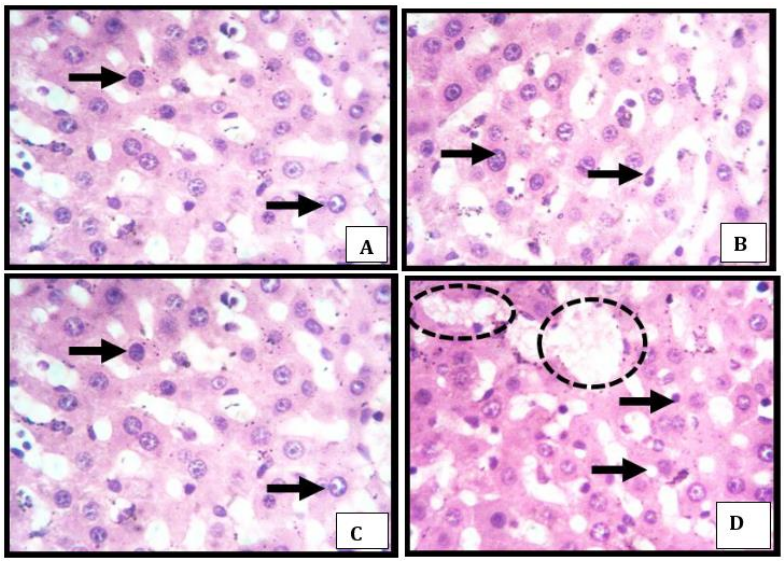

Figure 2. Histoarchitecture of the liver of experimental animals at a magnification (x400).

Black arrows represent inherent hepatocytes. Dotted black circles represent large vacuolation, while pink colorations represent fatty liver and bile plaques.

A: Liver of animals not exposed to rifampicin at all: It shows no histological distortion of the tissues. Liver histomorphology was normal, with hepatocytic nuclei well positioned in the cytoplasm.

B: The liver of animals exposed to rifampicin at 250 mg/kg bw without treatment showed cholestatic fatty liver.

C: The liver of animals exposed to rifampicin and treated with 100mg/kg bw of *P. daemia* showed unperturbed liver histomorphology with no histopathological distortion.

D: Liver of animals exposed to rifampicin and treated with silymarin at 100mg/kg bw showed intact liver tissue histomorphology with no histopathological distortion.

Serum level of creatine kinase, urea, and uric acid has been used as a diagnostic tool for measuring kidney integrity. In the present study, creatine kinase, urea, and uric acid increased significantly relative to control animals following exposure to rifampicin. This

observation suggests a free radical-induced upregulation in nitrogen metabolism. Consequently, there was an increase in the formation of urea and uric acid since the functional integrity of the glomerulus has been altered as a result of the oxidative attack on the kidney. These observations are consistent with the report of (Jaswal et al., 2013; Shukla et al., 2014). Treatment with graded doses of *P. daemia* caused restoration of creatine kinase, urea, and uric acid in a dose-dependent manner. The observed restoration can be linked to flavonoids and other antioxidant phytochemicals present in the extract, as earlier reported (Bhusari et al., 2018). It is also consistent with other reports on hepatoprotection (Renugadevi and Prabu, 2010; Renugadevi and Prabu, 2009).

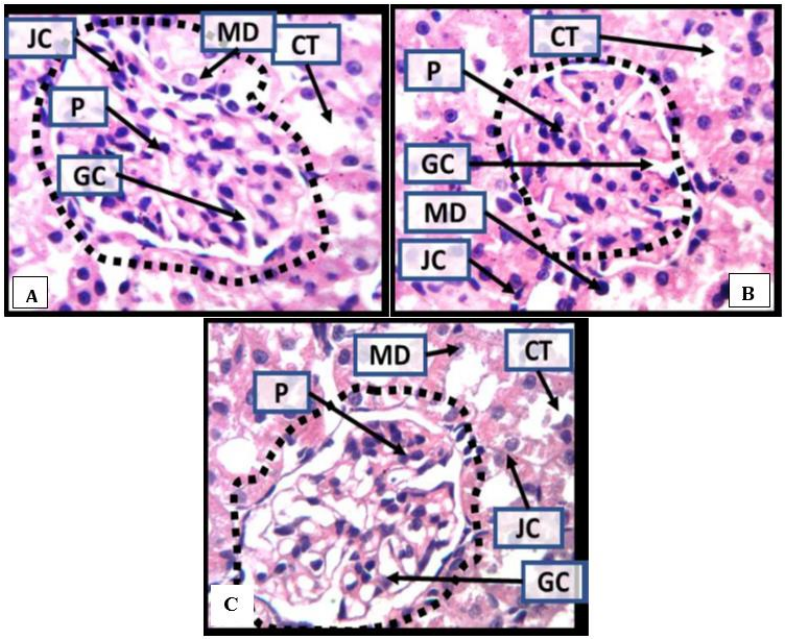

Figure 3. Histoarchitecture of the kidney tissue slice of experimental animals at a magnification (x400).
The black outline represents the renal corpuscle containing the glomerulus within the urinary space. Convoluted tubule (CT), glomerular capillaries (GC), and innate cells, including the intraglomerular podocytes (P) as well as the juxtaglomerular and macula densa cells.
A: Kidney slices of animals not exposed to rifampicin toxicity showed no noticeable histological distortion while the renal histoarchitecture was intact.
B: Kidney slice of animals exposed to rifampicin toxicity without treatment showed distorted renal histoarchitecture as well as glomerular atrophy. Degenerated renal tubules as well as intraluminal exfoliation, including nuclei pyknosis.
C: Kidney slice of animals exposed to rifampicin toxicity and treated with *P. daemia* at 100 mg/kg bw showed intact renal corpuscle having cellular delineation and appropriate distribution. There was no noticeable histopathological distortion.

Administration of rifampicin caused a significant derangement in lipid profile in experimental animals' liver, kidneys, and serum. Total cholesterol, triglycerides, and low-density lipoprotein (LDL) were markedly increased relative to control animals following exposure to rifampicin (Table 3a-c). This observation is in tandem with Tasduq et al. (2007), who showed that serum levels of triglyceride and cholesterol were significantly increased after administration of rifampicin to experimental rats. It also agrees with the earlier report of Santhosh et al. (2006), who observed a significant increase in serum LDL with a concomitant decrease in HDL-cholesterol following exposure of rats to rifampicin and isoniazid. When exposed animals were treated with graded doses of *P daemia*, there was a dose-dependent reversal of the toxicity imposed by rifampicin. In line with an earlier report, this reversal can be traced to antioxidant phytochemicals such as flavonoids and other polyphenols in the extract (Vaithiyanathan, 2015).

Moreover, rifampicin caused a significant decrease in the activities of SOD and CAT in the serum, liver, and kidney of experimental rats (Table 4a-c). Unarguably, SOD and CAT are the first lines of antioxidant enzymes' defense against oxidative stress. SOD is responsible for the catalytic dismutation of superoxide radicals to hydrogen peroxide. On the other hand, CAT is responsible for the catalytic decomposition of hydrogen peroxide into molecular oxygen and water (Lee and Sherman, 2000). Earlier reports suggested a decrease in antioxidant enzyme activity coupled with an increase in lipid peroxidation level after rifampicin intoxication (Sedlak and Snyder, 2003, Heit et al., 2017; Muller et al., 2006). Damage to renal tissue observed in the present study may be attributed to lipid peroxidation and inhibition of renal antioxidant enzymes following exposure to rifampicin.

Antioxidant capacity against reactive oxygen species is intrinsically linked to the enzymatic activity of SOD and catalase (Espinosa-Diez et al., 2015). Results from the present study indicate that rifampicin triggered a marked inhibition of SOD and CAT activity. This is probably due to the formation of potentially toxic intermediates. Inhibition of these enzymes led to an increase in the formation of superoxide anion radicals, eventually inactivating catalase and other hydrogen-peroxide-dependent enzymes (Naik and Panda, 2008). Oral administration of *P. daemia* leaf extracts markedly restored SOD and CAT activities. This observation can be linked to the free radical scavenging potentials of *P. daemia* due to flavonoids and other antioxidant phytochemicals in its leaf extract (Vaithiyanathan and Mirunalini, 2016). This observation is consistent with previous reports on *Solanum xanthocarpum* (Verma et al., 2015).

Reduced glutathione is a sulfhydryl tripeptide with potent antioxidant properties. Exposure to rifampicin caused a significant depletion in the GSH level. This observation partly suggests that the liver of exposed animals was vulnerable to free radicals attack caused by rifampicin. This also indicates the pivotal role of GSH in

cellular antioxidant defense. This observation is in agreement with Brehe and Burch (1976). Treatment with *P. daemia* led to an increase in GSH level, suggesting restoring antioxidant status. In agreement with an earlier report, this observation can be attributed to the antioxidant effect of the myriad of phytochemicals present in the leaf extract (Mohammed et al., 2004).

The significant decrease in the total protein of rifampicin-exposed rats agrees with Santhosh et al. (2006) and Eminzade et al. (2008). This observation could imply that toxic intermediates of rifampicin metabolism attacked the hepatocytes, thereby hampering the liver's ability to synthesize proteins such as albumin. Treatment with *P. daemia* significantly restored the protein level in a dose-dependent fashion. This observation agrees with Maheshwari and Vijayarengan (2021) and can be attributed to the antioxidant effect of phytochemicals present in the extract.

Lipid peroxidation was increased in the serum and organ homogenates of experimental rats exposed to rifampicin (Figure 1). This agrees with the report of Basheer et al. (2017), where administration of anti-tuberculosis drugs caused increased lipid peroxidation and the corresponding decrease in GSH levels. Perhaps, reactive intermediates of rifampicin metabolism bind covalently to critical macromolecules that overwhelm the endogenous antioxidant capacity, thereby triggering peroxidation of membrane lipids (Naik and Panda, 2008; Balakrishnan et al., 2012). The significant increase in lipid peroxidation suggests a compromise of the body's endogenous antioxidant capacity (Jaydeokar et al., 2014). The oral intervention of *P. daemia* leaf extract diminished the lipid peroxidation and restored the antioxidant status of the animals. Obviously, this is due to the array of polyphenols present in the extract. Specifically, this potential can be attributed to the condensed tannins and flavonoids present in *P. daemia* leaf extract, as earlier reported (Vaithiyanathan and Mirunalini, 2016).

Histological examination of liver and kidney of rifampicin–induced rats revealed many alterations such as tubular degeneration and atrophy of glomeruli (Figure 2A-D and 3A-C). Such observations were reported by Sidhu and Naugler (2012), who observed many histological and ultrastructural changes in the kidney of rats exposed to rifampicin.

4. Conclusions

The present study showed that aqueous extract of *P. daemia* leaf has an ameliorative effect on rifampicin-induced liver and kidney damage. All biochemical parameters, as well as histological evidence, established that the plant is a potential candidate that can be exploited in the management of liver and kidney diseases.

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Conflict of interest

All authors declare that there is no conflict of interest.

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Supplementary File

None.

References

Abirami, A., Nagarani, G., Siddhuraju, P., 2014. *In vitro* antioxidant, anti-diabetic, cholinesterase and tyrosinase inhibitory potential of fresh juice from *Citrus hystrix* and *C. maxima* fruits. *Food Science and Human Wellness*, 3(1), 16-25.

Antonyuk, S.V., Strange, R.W., Marklund, S.L., Hasnain, S.S., 2009. The structure of human extracellular copper–zinc superoxide dismutase at 1.7 Å resolution: insights into heparin and collagen binding. *Journal of Molecular Biology*, 388(2), 310-326.

Balakrishnan, S., Khurana, B.S., Singh, A., Kaliappan, I., Dubey, G.P., 2012. Hepatoprotective effect of hydroalcoholic extract of *Cissampelos pareira* against rifampicin and isoniazid induced hepatotoxicity. *Continental Journal of Food Science and Technology*, 6(1), 30-35.

Basheer, A.S., Siddiqui, A., Paudel, Y.N., Hassan, M.Q., Imran, M., Najmi, A.K., Akhtar, M., 2017. Hepatoprotective and antioxidant effects of fish oil on isoniazid-rifampin induced hepatotoxicity in rats. *PharmaNutrition*, 5(1), 29-33.

Beutler, E., 1963. Improved method for the determination of blood glutathione. *Journal of Laboratory and Clinical Medicine*, 61, 882-888.

Bhusari, S., Bhokare, S.G., Nikam, K.D., Chaudhary, A.N., Wakte, P.S., 2018. Pharmacognostic and Phytochemical investigation of stems of *Pergularia daemia*. *Asian Journal of Pharmacy and Pharmacology*, 4(4), 500-504.

Biour, J.M., Tymoczko, J.L., Stryer, L., 2004. Biochemistry. W.H. Freeman. pp. 656–660. ISBN 978-0-7167-8724-2.

Brehe, J.E., Burch, H.B., 1976. Enzymatic assay for glutathione. *Analytical Biochemistry*, 74(1), 189-197.

Byrne, J.A., Strautnieks, S.S., Mieli–Vergani, G., Higgins, C.F., Linton, K.J., Thompson, R.J., 2002. The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology*, 123(5), 1649-1658.

Capelle, P., Dhumeaux, D., Mora, M., Feldmann, G., Berthelot, P., 1972. Effect of rifampicin on liver function in man. *Gut*, 13(5), 366-371.

Chandak, R.R., 2010. Preliminary Phytochemical Investigation of *Pergularia daemia* linnInt. *Journal of Pharmaceutical Studies & Research*, 1(1), 11-16.

Chandak, R.R., Dighe, N.S., 2019. A Review on Phytochemical & Pharmacological Profile of *Pergularia daemia* linn. *Journal of Drug Delivery and Therapeutics*, 9(4-s), 809-814.

Dosumu, O.O., Ajetumobi, O.O., Omole, O.A., Onocha, P.A., 2019. Phytochemical composition and antioxidant and antimicrobial activities of *Pergularia daemia*. *Journal of Medicinal Plants for Economic Development*, 3(1), 1-8.

Eminizade, N., Krance, S.M., Notenboom, S., Shi, S., Tieu, K., Hammond, C.L., 2008. Glutathione dysregulation and the etiology and progression of human diseases. *Biological Chemistry*, 390(3), 191-214.

Englehardt, A., 1970. Measurement of alkaline phosphatase. *Aerztl Labor*, 16(42), 1.

Espinosa-Diez, C., Miguel, V., Mennerich, D., Kietzmann, T., Sánchez-Pérez, P., Cadenas, S., Lamas, S., 2015. Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biology*, 6, 183-197.

Friedewald, W.T., Levy, R.I., Fredrickson, D.S., 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry*, 18, 499–502.

Grosset, J., Leventis, S., 1983. Adverse effects of rifampin. *Reviews of Infectious Diseases*, 5(Supplement_3), S440-S446.

Grove, T.H., 1979. Effect of reagent pH on determination of high-density lipoprotein cholesterol by precipitation with sodium phosphotungstate-magnesium. *Clinical Chemistry*, 25(4), 560-564.

Heit, C., Marshall, S., Singh, S., Yu, X., Charkoftaki, G., Zhao, H., Vasiliou, V., 2017. Catalase deletion promotes prediabetic phenotype in mice. *Free Radical Biology and Medicine*, 103, 48-56.

Jaswal, A., Sinha, N., Bhadauria, M., Shrivastava, S., Shukla, S., 2013. Therapeutic potential of thymoquinone against anti-tuberculosis drugs induced liver damage. *Environmental Toxicology and Pharmacology*, 36(3), 779-786.

Jaydeokar, A.V., Bandawane, D.D., Bibave, K.H., Patil, T.V., 2014. Hepatoprotective potential of *Cassia auriculata* roots on ethanol and antitubercular drug-induced hepatotoxicity in experimental models. *Pharmaceutical Biology*, 52(3), 344-355.

Jelkmann, W., 2001. The role of the liver in the production of thrombopoietin compared with erythropoietin. *European Journal of Gastroenterology & Hepatology*, 13(7), 791-801.
Kim, J.H., Nam, W.S., Kim, S.J., Kwon, O.K., Seung, E.J., Jo, J.J., Lee, S., 2017. Mechanism investigation of rifampicin-induced liver injury using comparative toxicoproteomics in mice. *International Journal of Molecular Sciences*, 18(7), 1417.
Kohli, H.S., Bhaskaran, M.C., Muthukumar, T., Thennarasu, K., Sud, K., Jha, V., Sakhuja, V., 2000. Treatment-related acute renal failure in the elderly: a hospital-based prospective study. *Nephrology Dialysis Transplantation*, 15(2), 212-217.
Kosanam, S., Boyina, R., 2015. Drug-induced liver injury: A review. *International Journal of Pharmacological Research*, 5(2), 24-30.
Krishnaiah, D., Sarbatly, R., Nithyanandam, R., 2011. A review of the antioxidant potential of medicinal plant species. *Food and Bioproducts Processing*, 89(3), 217-233.
Larrey, D., 2000. Drug-induced liver diseases. *Journal of Hepatology*, 32, 77-88.
Lee, C.L., Sherman, P.M., 2000. Pediatric Gastrointestinal Disease. Connecticut: PMPH-USA. p. 751. ISBN 978-1-55009-364-3.
Maheshwari, M., Vijayarengan, P., 2021. Phytochemical Evaluation, FT-IR and GC-MS Analysis of Leaf Extracts of *Pergularia daemia*. *Nature Environment and Pollution Technology*, 20(1), 259-265.
Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry*, 247(10), 3170-3175.
Mohammed, S., Kasera, P.K., Shukla, J.K., 2004. Unexploited plants of potential medicinal value from the Indian Thar desert. *Natural Product Radiance*, 3, 69-74.
Muller, F.L., Song, W., Liu, Y., Chaudhuri, A., Pieke-Dahl, S., Strong, R., Van Remmen, H., 2006. Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. *Free Radical Biology and Medicine*, 40(11), 1993-2004.
Naik, S.R., Panda, V.S., 2008. Hepatoprotective effect of Ginkgoselect Phytosome® in rifampicin induced liver injurym in rats: Evidence of antioxidant activity. *Fitoterapia*, 79(6), 439-445.
Nithyatharani, R., Kavitha, U., 2018. Phytochemical Studies on the Leaves of *Pergularia daemia* Collected from Villupuram District, Tamil Nadu, India. *IOSR Journal of Pharmacy*, 8(1), 9-12.
Panich, U., Onkoksoong, T., Limsaengurai, S., Akarasereenont, P., Wongkajornsilp, A., 2012. UVA-induced melanogenesis and modulation of glutathione redox system in different melanoma cell lines: the protective effect of gallic acid. *Journal of Photochemistry and Photobiology B: Biology*, 108, 16-22.
Rana, S.V., Pal, R., Vaiphie, K., Singh, K., 2006. Effect of different oral doses of isoniazid-rifampicin in rats. *Molecular and Cellular Biochemistry*, 289(1), 39-47.
Reitman, S., Frankel, S., 1957. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *American Journal of Clinical Pathology*, 28(1), 56-63.
Renugadevi, J., Prabu, S.M., 2009. Naringenin protects against cadmium-induced oxidative renal dysfunction in rats. *Toxicology*, 256(1-2), 128-134.
Renugadevi, J., Prabu, S.M., 2010. Cadmium-induced hepatotoxicity in rats and the protective effect of naringenin. *Experimental and Toxicologic Pathology*, 62(2), 171-181.
Santhosh, S., Sini, T.K., Anandan, R., Mathew, P.T., 2006. Effect of chitosan supplementation on antitubercular drugs-induced hepatotoxicity in rats. *Toxicology*, 219(1-3), 53-59.
Saukkonen, J.J., Cohn, D.L., Jasmer, R.M., Schenker, S., Jereb, J.A., Nolan, C.M., 2006. On the behalf of ATS (American Thoracic Society). Hepatotoxicity of Antituberculosis Therapy. *American Journal of Respiratory and Critical Care Medicine*, 174(8), 935-952.
Sedlak, T.W., Snyder, S.H., 2004. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics*, 113(6), 1776-1782.
Sentman, M.L., Granström, M., Jakobson, H., Reaume, A., Basu, S., Marklund, S.L., 2006. Phenotypes of mice lacking extracellular superoxide dismutase and copper-and zinc-containing superoxide dismutase. *Journal of Biological Chemistry*, 281(11), 6904-6909.
Sharma, R., Sharma, V.L., 2015. Review: treatment of toxicity caused by anti-tubercular drugs by use of different herbs. *International Journal of Pharma Sciences and Research*, 6(10), 1288-1294.
Shukla, S., Sinha, N., Jaswal, A., 2014. Anti Oxidative, Anti Peroxidative and Hepatoprotective Potential of *Phyllanthus amarus* Against Anti Tb Drugs. In Pharmacology and Nutritional Intervention in the Treatment of Disease. *IntechOpen*, 2014, 283-294.
Sidhu, D., Naugler, C., 2012. Fasting time and lipid levels in a community-based population: a cross-sectional study. *Archives of Internal Medicine*, 172(22), 1707-1710.
Sinha, A.K., 1972. Colorimetric assay of catalase. *Analytical Biochemistry*, 47(2), 389-394.
Tasduq, S.A., Kaiser, P., Sharma, S.C., Johri, R.K., 2007. Potentiation of isoniazid-induced liver toxicity by rifampicin in a combinational therapy of antitubercular drugs (rifampicin, isoniazid and pyrazinamide) in Wistar rats: A toxicity profile study. *Hepatology Research*, 37(10), 845-853.
Tietz, N.W., 1995. Clinical Guide to Laboratory Tests, 3rd Edition, W.B. Saunders, Philadelphia.
Trinder, P., 1969. A simple Turbidimetric method for the determination of serum cholesterol. *Annals of Clinical Biochemistry*, 6(5), 165-166.
Ueno, Y., Kizaki, M., Nakagiri, R., Kamiya, T., Sumi, H., Osawa, T., 2002. Dietary glutathione protects rats from diabetic nephropathy and neuropathy. *The Journal of Nutrition*, 132(5), 897-900.
Vaithiyanathan, V., Mirunalini, S., 2015. Quantitative variation of bioactive phyto compounds in ethyl acetate and methanol extracts of *Pergularia daemia* (Forsk.) Chiov. *Journal of Biomedical Research*, 29(2), 169-172.
Vaithiyanathan, V., Mirunalini, S., 2016. Assessment of anticancer activity: A comparison of dose–response effect of ethyl acetate and methanolic extracts of *Pergularia daemia* (Forsk). *Oral Science International*, 13(1), 24-31.
Vanderlinde, R.E., 1981. Urinary enzyme measurements in the diagnosis of renal disorders. *Annals of Clinical & Laboratory Science*, 11(3), 189-201.
Verma, P., Paswan, S., Singh, S.P., Shrivastva, S., Rao, C.V., 2015. Assessment of hepatoprotective potential of *Solanum xanthocarpum* (whole plant) Linn. against isoniazid & rifampicin induced hepatic toxicity in Wistar rats. *Indian Journal of Research in Pharmacy and Biotechnology*, 3, 373-379.
Weichselbaum, C.T., 1946. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. *American Journal of Clinical Pathology*, 16(3_ts), 40-49.
Yue-Ming, W., Sergio, C.C., Christopher, T.B., Taoshen, C., 2014. Pregnane X receptor and drug-induced liver injury expert Opin. *Journal of Drug Metabolism & Toxicology*, 10(11), 1521-1532.

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RESEARCH ARTICLE

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HPLC profile of phenolic acids and flavonoids of *Ocimum sanctum* and *O. basilicum*

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ABSTRACT

This study aimed to investigate the antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC), as well as the phenolic profile of two species of *O. sanctum* (OS) and *O. basilicum* (OB). The TPC, TFC, and cultivated *sanctum*'s phenolic profiles were similar. The TPC of OS and OB produced 386 and 383 mg gallic acid equivalent (GAE) per 100 g, whereas 201 and 203 mg quercetin equivalent (QE) per 100 g of the extract was obtained during the TFC assay. The antioxidant activity of the extracts was determined by scavenging of DPPH radicals with an inconsiderable difference. HPLC techniques separated the individual phenolic acids and flavonoids. Phenolic acids (gallic, caffeic, ferulic, sinapic, and syringic) and flavonoids (quercetin, luteolin, rutin, apigenin, and kaempferol) were commonly identified and quantified in the chromatogram of OS and OB. The maximum gallic acid and quercetin content were found among phenolic acids and flavonoids. The maximum yield of quercetin was analyzed in both extracts.

1. Introduction

Ocimum sanctum and *O. basilicum* belong to the Lamiaceae family; both are used as ornamental and culinary herbs. The genus *Ocimum* contains more than 150 species of herbs found in tropical regions of Asia and are rich sources of phytochemicals that contribute to many putative health benefits (Ali et al., 2014; Malekshahi et al., 2021; Mokat and Kharat, 2022; Shoker et al., 2021). Traditionally, it is used as a medicinal plant to treat cough, diarrhea, warts, worm, and kidney malfunctions. Externally, it is used as an ointment for insect bites and in the treatment of acne due to having antibacterial, anti-carcinogenic, antimutagenic, and antioxidant activity (Anantharam and Chittibabu, 2021; de Lima et al., 2014; Pandey et al., 2015; Sayyad et al., 2022; Shafqatullah et al., 2013). Numerous studies signify the strong antioxidant and biological activities of *Ocimum,* among other medicinal plants. The previous literature shows that Ocimum has a remarkable phenolic content yield (Gavrić et al., 2018; Kelm et al., 2000; Samson et al., 2007).

Phenolic compounds are the secondary plant metabolites that are widely distributed in plants. These compounds play an important role in health-promoting factors (Khatri et al., 2019; Scheublin et al., 2014). Scientific research suggests that these compounds have antioxidant properties that trap free radicals and reduce the risk of cancer, heart, and chronic diseases (Imram et al., 2014; Ssepuuya et al., 2021; Troise et al., 2014). These phenolic compounds have more than dozen of sub-classes but are mainly classified as simple and polyphenol (Matulja et al., 2022).

Phytonutrients like flavonoids have beneficial anti-inflammatory effects, and they protect your cells from oxidative damage that can lead to disease. These dietary antioxidants can prevent the

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development of cardiovascular disease, diabetes, cancer, and cognitive diseases like Alzheimer's and dementia.

Similarly, flavonoids, flavonoid polymers, and phenolic acids are most well-known due to having many advantageous features in the area where highly antioxidants and anti-inflammatory therapy are required to avoid diseases including cardiovascular diseases, diabetes, cancer, and cognitive diseases like Alzheimer's, and dementia (Khatri et al., 2019; Nemzer et al., 2021). These compounds are essential antioxidants due to their redox potential, which acts as a strong reducing agent, hydrogen donors, and single oxygen quenchers (Pisoschi et al., 2021). These compounds also possess metal chelating properties (Javanmardi et al., 2003; Qureshi et al., 2014; Tatipamula and Kukavica, 2021).

Due to many phenolic compounds in plants, quantifying and identifying each phenolic acid and flavonoid is tedious. Different techniques have been used for the separation and quantification of these compounds, wherein thin layer chromatography (TLC) and high-performance liquid chromatographic (HPLC) are the most widely used (Montedoro et al., 1992; Proestos et al., 2005; Qureshi et al., 2014).

The current study was designed to evaluate the TPC, TFC, and antioxidant activity of methanol extracts (obtained by cold extraction method) of *O. sanctum* and *O. basilicum* as well as the profile of phenolic acids and flavonoids using the HPLC technique.

Economic feasibility, extremely quick and efficient HPLC technique used in this increases the worth of the current study compared to some previously reported methods, such as TLC and HPTLC, etc.

2. Materials and methods

2.1. Collection of samples and materials

Greenish stems and leaves of the plant materials were collected separately from the botanical garden of PCSIR Labs complex Peshawar, brought to the Laboratory, washed thoroughly with tap water, and shade dried at room temperature. All solvents were of analytical grade and used as received.

2.2. Experimental procedure

The samples were extracted with methanol following a previously reported method with a slight modification (Khattak et al., 2007). Briefly, the collected leaves were air-dried at room temperature. Then, a 100 g powder sample was extracted with 98.8% methanol on an orbital shaker for 3 hours. The extract was then centrifuged at 6.000 rpm for 10 min. The supernatant was collected, and the organic solvent was removed by a sample concentrator in the presence of N_2 gas. The residues were then re-dissolved in 10 ml of methanol and stored for further analysis.

2.3. Determination of antioxidant activity

The antioxidant activity of OS and OB methanol extracts was determined by DPPH radical scavenging activity (Ashif and Ullah, 2013). 1 ml of each extract was diluted with 9 ml methanol and centrifuged for 5 min at 6.000 rpm. 1ml of the supernatant was added with 2 ml of DPPH methanol solution (0.004%, w/v). The absorbance was measured at 517 nm against the blank after 30 minutes of incubation. The scavenging activity of the extract was determined in percent using the following equation.

$$\%\ Scavenging\ Activity = \frac{Abs\ of\ control\ -\ Abs\ of\ sample}{Abs\ of\ the\ control} \times 100$$

2.4. Determination of total phenolic content (TPC)

The TPC of methanol extract OS and OB was carried out using Folin-Ciocalteau Reagent (FCR) (Meda et al., 2005). 100 µg extract was diluted with 400 µl of deionized water and 2ml of 7.5 (m/v) sodium carbonate solution. Then, the resultant solutions were mixed with 2.5 ml of 0.2M Folin-Ciocalteu reagent and incubated for 2h. The absorbance was measured against blank at 760 nm using a UV/Vis spectrophotometer, and the TPC was measured from the standard curve of six different concentrations of gallic acid. The result was expressed as mg of gallic acid equivalents per gram dry weight of leaves.

2.5. Determination of total flavonoid content (TFC)

The TFC was analyzed by the spectrophotometric method (Boateng et al., 2008). 1 ml of each methanol extract was diluted with 4 ml of deionized water and 300 µl of sodium nitrate solution (15 g / 100 ml), 4 ml of sodium hydroxide solution (4 mg / 100 ml), and 300 µl of methanol aluminum chloride (10 g / 100 ml) was added and made the final solution up to 10 ml with distilled water. The absorbance was then measured at 510 nm after 15 minutes. This method is based on the reaction between aluminum chloride and flavonoids. The TFC was measured from the standard curve of six different concentrations of quercetin and expressed as mg of quercetin equivalent per gram of dry weight.

$$Y = 0.052x + 0.049, R_2 = 0.999$$

2.6. Determination of phenolic acids and flavonoids by HPLC

Determination of phenolic acids and flavonoids was carried out by HPLC (Hitachi D-2000) using a UV-Vis detector (L-2420). The phenolic acids (gallic, syringic, vanillic, and caffeic acid) were detected at 280 nm while flavonoids (quercetin, luteolin, rutin, apigenin, and kaempferol) at 360 nm according to the reported method (Pająk et al., 2014). The chromatographic separation was achieved using Intersil column ODS-3 C18 (5 µm, 250 × 4.6 mm GL Science Inc. Tokyo, Japan) at 30 °C. The chromatographic analysis was conducted with gradient elution using two solvents. Solvent-A: acidified water (2.5 g Acetic acid / 100ml) and Solvent-B: methanol. A linear gradient was applied for the first 10 minutes, with an increase of mobile phase B from 3 to 8%. After 10 minutes, the mobile phase B increased to 15, 20, 30, and 40% at 20, 30, 40, and 50 minutes, respectively. The quantification of phenolic acid and flavonoids was carried out using the external standard method.

3. Results and discussion

3.1. Antioxidant activity, total phenolic, and total flavonoid contents

The antioxidant activity, TPC, and TFC of OS and OB are given in Table 1. The content was evaluated by scavenging of free radical of DPPH. The antioxidant molecules in the extract quench these free radicals and convert them to colorless 2,2 diphenyl-1-hydrazine resulting in the decrease in absorbance of the DPPH solution. The OS and OB scavenged 61 and 60% of the DPPH free radicals.

The TPC and TFC of *O. sanctum* and *O. basilicum* are determined as GAE and QE, presented in Table 1. The TPC content was found as 386 mg GAE/100 g in *O. sanctum* and 383 mg GAE/100 g in *O. basilicum*, while the TFC was 201.6 and 203.4 mg QE/100 g. Plants

are a good source of phenolic compounds, and the study by Javanmardi et al. (2003) showed that the TPC of 20 medicinal plants was in the range of 2.34-152.32 mg GAE/100g. However, we found TPC too high in both *Ocimum* species compared to the abovementioned authors. These results agree with Naithani et al. (2006) and Kähkönen et al. (1999) that the TPC and TFC depend upon the extraction procedure and solvent. The authors found that these two *Ocimum* species have a very close relation to the phytochemicals.

Table 1. Antioxidant activity, total phenolic and flavonoid contents of *O. sanctum* and *O. basilicum*

Sample	% Scavenging (DPPH)	Content (mg / 100 g)	
		Total phenolic	Total flavonoid
O. sanctum	61.4 ± 1.2*	386 ± 2.5	201.6 ± 1.32
O. basilicium	60.56 ± 1.5	383 ± 2.4	203.4 ± 1.43

*RSD: relative standard deviation; the number of replicates: *n* = 3

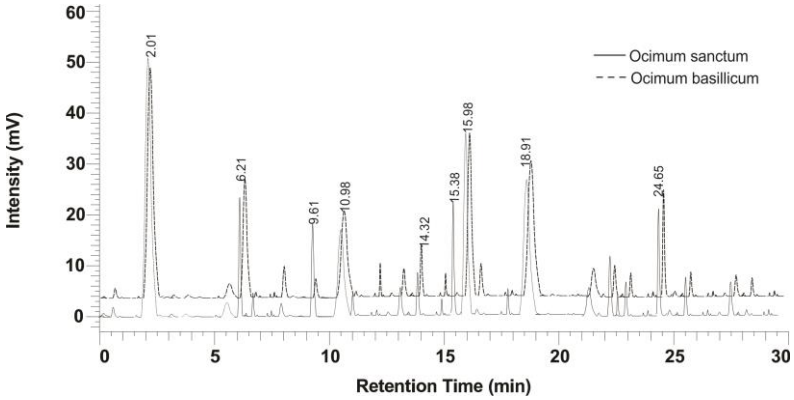

Figure 1. HPLC profile of OS and OB [Detection: 230 nm, Peaks at retention time: 2.01 (gallic); other unidentified compounds]

Table 2. Phenolic acid profile of *O. sanctum* and *O. basilicum*

Sample	Phenolic acid (mg / 100 g)					Total
	Gallic	Caffeic	Ferulic	Sinapic	Syringic	
O. sanctum	5.5 ± 0.8*	8.1 ± 0.6	5.3 ± 1.2	3.4 ± 0.8	2.1 ± 0.5	56.4
O. basilicum	6.5 ± 1.0	7.9 ± 0.5	4.9 ± 1.9	4.6 ± 0.7	1.8 ± 0.3	58.1

*RSD: relative standard deviation; the number of replicates: *n* = 3

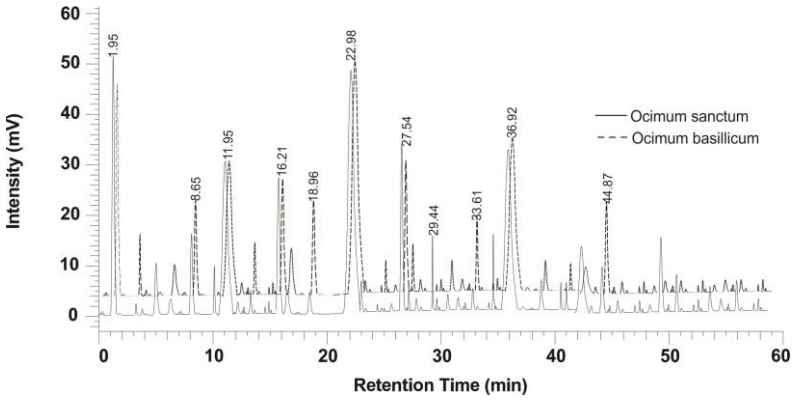

Figure 2. HPLC profile of OS and OB [Detection: 230 nm, Peaks at retention time: 2.01 (gallic); other unidentified compounds]

Table 3. Flavonoid profile of *O. sanctum* and *O. basilicum*

Sample	Flavonoid (mg / 100 g)					Total
	Quercetin	Luteolin	Rutin	Apigenin	Kaempferol	
O. sanctum	2.1 ± 0.3*	1.6 ± 0.7	1.6 ± 0.2	1.8 ± 0.1	1.9 ± 0.1	19.0
O. basilicum	3.4 ± 0.3	1.5 ± 0.5	1.9 ± 0.1	2.3 ± 0.2	1.1 ± 0.2	19.2

*RSD: relative standard deviation; the number of replicates: *n* = 3

3.2. The profile of phenolic acids and flavonoids

In this study, free phenolic acids were extracted and determined in *O. sanctum* and *O. basilicum*. Some other phenolic acids, such as esters, glycosides, and bound complexes, are also present in plants. Five phenolic acids (gallic, caffeic, sinapic, ferulic, and syringic) were identified in the HPLC chromatogram shown in Figure 1, and the quantitative analysis is presented in Table 2. The caffeic and gallic acid content was found in maximum yield in both the samples (8.1 and 5.5 mg / 100 g in *O. sanctum*, while 6.5 and 7.9 mg / 100 g in *O.*

basilicum). The ferulic, chlorogenic, and syringic content was 5.3, 3.4, and 2.1 mg / 100 g in *O. sanctum* and 4.9, 4.6, and 1.8 mg / 100 g in *O. basilicum*, respectively.

In our previous study, we had identified and quantified the five phenolic acids, including gallic and syringic, in methanol extract of *O. sanctum* by different extraction procedures and methods, in which syringic acid was not detected while gallic acid content was 2.97 and chlorogenic was 1.86 mg / 100 g, which is in close agreement with a previous study (Jayasinghe et al., 2003). Using the aforementioned method, sinapic acid was not detected in any sample. On the other hand, five flavonoids were identified in the HPLC chromatogram shown in Figure 2, and the content of flavonoids was quantified in Table 3.

The quercetin content was found to be maximum in both samples, 2.1 mg / 100 g in *O. sanctum* and 3.4 mg / 100 g in *O. basilicum*, while the rest of luteolin, rutin, apigenin, and kaempferol were 1.6, 1.6, 1.8, and 1.9 mg /100 g in *O. sanctum* and 1.5, 1.9, 2.3, and 1.1 mg / 100 g in *O. basilicum* (Lee and Scagel, 2009).

4. Conclusions

This protocol represents a comparative study of natural antioxidants, for example, phenolic acid and flavonoid content determination of two potential medicinal plants. Both OS and OB were potential sources of natural antioxidants. The plants were rich in active components such as phenolic acids, gallic, caffeic, ferulic, sinapic, syringic, and flavonoids, including quercetin, luteoline, and rutin apigenin, and kaempferol. The TPC content was 386 mg GAE / 100 g in *O. sanctum* and 383 mg GAE / 100 g in *O. basilicum*, whereas the TFC was 201.6 and 203.4 mg QE / 100 g on average. Further, OS and OB scavenged 61% and 60% of the DPPH free radicals. As previously described, syringic acid was not detected in our previous investigations, although the used protocols were considerably identical. Therefore, further analysis is suggested to verify and purify the biologically active components of the plants above to optimize the production of therapeutic contents for their ultimate implementation in the food industry, such as herbal antioxidants, etc.

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None.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

Shafqat Ullah: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing, Review & Editing
Naseem Rauf: Data curation, Funding acquisition, Project administration, Software, Validation, Writing
Arshad Hussain: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Writing, Review & Editing
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Supplementary File

None.

References

Ali, R., Chauhan, V., Farooq, S., Khan, A., Farooq, U., 2014. *In vitro* analysis of antibacterial activity of *Ocimum sanctum* against pathogenic bacteria and quantification of ursolic acid and oleanolic acid. *International Journal of Pharmaceutical Sciences Review & Research*, 25, 13-17.

Anantharam, G., Chittibabu, C.V., 2021. Herbal Gardens of Educational Institutions and Entrepreneurship Development: A Case Study from south India. *Center for Development Economic*, 8(10), 62-84.

Ashif, M.K., Ullah, S., 2013. Chemical composition and minerals analysis of *Hippophae rhamnoides*, *Azadirachta indica*, *Punica granatu* and *Ocimum sanctum* leaves. *World Journal of Dairy & Food Sciences*, 8(1), 67-73.

Boateng, J., Verghese, M., Walker, L.T., Ogutu, S., 2008. Effect of processing on antioxidant contents in selected dry beans (*Phaseolus* spp. L.). *LWT-Food Science and Technology*, 41(9), 1541-1547.

de Lima, V.T., Vieira, M.C., Kassuya, C.A.L., Cardoso, C.A.L., Alves, J.M., Foglio, M.A., Formagio, A.S.N., 2014. Chemical composition and free radical-scavenging, anticancer and anti-inflammatory activities of the essential oil from *Ocimum kilim* and *scharicum*. *Phytomedicine*, 21(11), 1298-1302.

Gavrić, T., Jurković, J., Hamidović, S., Haseljić, S., Lalević, B., Čorbo, A., Bezdrob, M., 2018. Yield and contents of some bioactive components of basil (*Ocimum basilicum* L.) depending on time of cutting. *Studia Universitatis Vasile Goldis Seria Stiintele Vietii (Life Sciences Series)*, 28(4), 192-197.

Imram, I., Zia-Ul-Haq, M., Calani, L., Mazzeo, T., Pellegrini, N., 2014. Phenolic profile and antioxidant potential of selected plants of Pakistan. *Journal of Applied Botany and Food Quality*, 87, 30-35.

Javanmardi, J., Stushnoff, C., Locke, E., Vivanco, J.M., 2003. Antioxidant activity and total phenolic content of Iranian *Ocimum accessions*. *Food Chemistry*, 83(4), 547-550.

Jayasinghe, C., Gotoh, N., Aoki, T., Wada, S., 2003. Phenolics composition and antioxidant activity of sweet basil (*Ocimum basilicum* L.). *Journal of Agricultural and Food Chemistry*, 51(15), 4442-4449.

Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S., Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, 47(10), 3954-3962.

Kelm, M.A., Nair, M.G., Strasburg, G.M., DeWitt, D.L., 2000. Antioxidant and cyclooxygenase inhibitory phenolic compounds from *Ocimum sanctum* Linn. *Phytomedicine*, 7(1), 7-13.

Khatri, S., Paramanya, A., Ali, A., 2019. Phenolic acids and their health-promoting activity. *Plant and Human Health*, Volume 2 (pp. 661-680), Springer.

Khattak, A.B., Zeb, A., Bibi, N., Khalil, S.A., Khattak, M.S., 2007. Influence of germination techniques on phytic acid and polyphenols content of chickpea (*Cicer arietinum* L.) sprouts. *Food Chemistry*, 104(3), 1074-1079.

Lee, J., Scagel, C. F. (2009). Chicoric acid found in basil (*Ocimum basilicum* L.) leaves. *Food Chemistry*, 115(2), 650-656.

Malekshahi, F., Mehrabi, A.A., Tavakol, E., Mehdikhanlo, K., Shariati, V., 2021. The Comparison of the Essential Oil Percentage Content and Composition of African Basil (*Ocimum gratissimum* L.) in Spring and Fall Harvests. *Journal of Horticultural Science*, 35(2), 209-221.

Matulja, D., Vranješević, F., Kolympadi Markovic, M., Pavelić, S.K., Marković, D., 2022. Anticancer Activities of Marine-Derived Phenolic Compounds and Their Derivatives. *Molecules*, 27(4), 1449.

Meda, A., Lamien, C.E., Romito, M., Millogo, J., Nacoulma, O.G., 2005. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry*, 91(3), 571-577.

Mokat, D.N., Kharat, T.D., 2022. Essential Oil Composition in Leaves of *Ocimum* Species Found in Western Maharashtra, India. *Journal of Essential Oil Bearing Plants*, 25(1), 1-8.

Montedoro, G., Servili, M., Baldioli, M., Miniati, E., 1992. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *Journal of Agricultural and Food Chemistry*, 40(9), 1571-1576.

Naithani, V., Nair, S., Kakkar, P., 2006. Decline in antioxidant capacity of Indian herbal teas during storage and its relation to phenolic content. *Food Research International*, 39(2), 176-181.

Nemzer, B., Kalita, D., Yashin, A.Y., Yashin, Y.I., 2021. Chemical Composition and Polyphenolic Compounds of Red Wines: Their Antioxidant Activities and Effects on Human Health—A Review. *Beverages*, 8(1), 1.

Pająk, P., Socha, R., Gałkowska, D., Rożnowski, J., Fortuna, T., 2014. Phenolic profile and antioxidant activity in selected seeds and sprouts. *Food Chemistry*, 143, 300-306.
Pandey, H., Pandey, P., Singh, S., Gupta, R., Banerjee, S., 2015. Production of anti-cancer triterpene (betulinic acid) from callus cultures of different *Ocimum* species and its elicitation. *Protoplasma*, 252(2), 647-655.
Pisoschi, A.M., Pop, A., Iordache, F., Stanca, L., Predoi, G., Serban, A.I., 2021. Oxidative stress mitigation by antioxidants-an overview on their chemistry and influences on health status. *European Journal of Medicinal Chemistry*, 209, 112891.
Proestos, C., Chorianopoulos, N., Nychas, G.J., Komaitis, M., 2005. RP-HPLC analysis of the phenolic compounds of plant extracts. Investigation of their antioxidant capacity and antimicrobial activity. *Journal of Agricultural and Food Chemistry*, 53(4), 1190-1195.
Qureshi, M.N., Stecher, G., Bonn, G.K., 2014. Determination of total polyphenolic compounds and flavonoids in Juglans regia leaves. *Pakistan Journal of Pharmaceutical Sciences*, 27(4), 865-869.
Samson, J., Sheeladevi, R., Ravindran, R., 2007. Oxidative stress in brain and antioxidant activity of *Ocimum sanctum* in noise exposure. *Neurotoxicology*, 28(3), 679-685.
Sayyad, S.S., Patil, B.D., Dhumal, V.S., 2022. Evaluation of physico-chemical properties of Lassi prepared with optimized level of Tulsi or Basil (*Ocimum sanctum* L.). *The Pharma Innovation Journal*, 11(1), 285-288.
Scheublin, T.R., Deusch, S., Moreno-Forero, S.K., Müller, J.A., van der Meer, J.R., Leveau, J.H., 2014. Transcriptional profiling of Gram-positive *Arthrobacter* in the phyllosphere: induction of pollutant degradation genes by natural plant phenolic compounds. *Environmental Microbiology*, 16(7), 2212-2225.
Shafqatullah, M.K., Asadullah, K., Khan, F.A., 2013. Comparative analyses of *Ocimum santum* stem and leaves for phytochemicals and inorganic constituents. *Middle-East Journal of Scientific Research*, 13(2), 236-240.
Shoker, R.M., Raheema, R.H., Shamkhi, I.J., 2021. Antimicrobial activity, HPLC analysis of phenolic extract of *Ocimum basilicum* and *Ocimum sanctum*. *Biochemical and Cellular Archives*, 21(2), 1-8.
Ssepuuya, G., Kagulire, J., Katongole, J., Kabbo, D., Claes, J., Nakimbugwe, D., 2021. Suitable extraction conditions for determination of total anti-oxidant capacity and phenolic compounds in *Ruspolia differens* Serville. *Journal of Insects as Food and Feed*, 7(2), 205-214.
Tatipamula, V.B., Kukavica, B., 2021. Phenolic compounds as antidiabetic, anti-inflammatory, and anticancer agents and improvement of their bioavailability by liposomes. *Cell Biochemistry and Function*, 39(8), 926-944.
Troise, A.D., Ferracane, R., Palermo, M., Fogliano, V., 2014. Targeted metabolite profile of food bioactive compounds by Orbitrap high resolution mass spectrometry: the "FancyTiles" approach. *Food Research International*, 63, 139-146.

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RESEARCH ARTICLE

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Antihemolytic activity of hydroalcoholic leaves and bark extracts from *Rhamnus alaternus* against AAPH induced hemolysis on human erythrocytes

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ABSTRACT

Rhamnus alaternus is a Mediterranean shrub that has been used in traditional medicine to treat various diseases. This study aimed to determine the phenolic composition, as well as antioxidant and antihemolytic activities of *R. alaternus* leaves (LRA) and bark (BRA), extracts against AAPH-induced hemolysis. The extraction yields were 19.8% and 18.2% for leaves and bark. Total polyphenols (88.1 ± 1.83 mg GAE/g) and condensed tannins (36.24 ± 5.44 mg CE/g) were higher in BRA extract than in LRA extract (80.22 ± 1.4 mg GAE/g and 23.48 ± 0.25 mg CE/g, respectively). However, LRA extract was found to be richer in total flavonoids (64.6 ± 2.6 mg QE/g) and flavones/flavonol (18.34 ± 1.65 mg QE/g) than BRA extract (39.87 ±0.58 mg QE/g and 10.08 ± 0.35 mg QE/g), respectively. The IC_{50} of DPPH and ABTS radical scavenging activity were 86.59 ± 2 µg/ml and 12.49 ± 0.29 µg/ml for LRA extract and 69.23 ± 2.14 µg/ml and 12.83 ± 0.13 µg/ml for BRA extract, respectively. Also, both extracts showed good reducing power with 157.09 ± 5.53 mg Asc E/g for LRA extract and 194.97 ± 1.46 mg Asc E/g for BRA extract. The hemolytic effect was tested on human erythrocytes, and both extracts did not have cytotoxic effects at low doses. To induce hemolysis, AAPH was used at a concentration of 200 mM with an incubation time of 4h. The antihemolytic activity of the two extracts showed that pretreatment of human erythrocytes with various doses significantly reduced AAPH-induced hemolysis in a dose-dependent manner. Indeed, at 200 µg/ml, the percentages of hemolysis inhibition were 99.41 ± 1.17% and 76.26 ± 12.03% for BRA and LRA extracts, respectively. BRA extract was more effective (IC_{50} = 106.70 ± 1.48 µg/ml) compared to LRA extract (IC_{50} = 148.64 ± 7.04 µg/ml). Our results demonstrate for the first time that *R. alaternus* attenuates AAPH-induced hemolysis and can be used to prevent and treat hemolytic anemias.

1. Introduction

Medicinal plants are the primary reservoir of indigenous medical systems. Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve plant extracts (Abate, 2019). Nowadays, plants are of increasing interest due to their content of secondary metabolites known to have antioxidant activity and potency that can protect the body's cells from oxidative stress and free radical damage (Chansiw et al., 2018). Indeed, cells contain antioxidant systems that protect them from deleterious effects and regulate the generation of free radicals through diverse mechanisms (Fibach and Rachmilewitz, 2008). However, these systems can be overwhelmed in case of excess free radicals.

Oxidative stress is involved in apoptosis and cellular aging but also hemolytic anemia. The latter is an indicator of free radical damage to the red blood cell membrane (RBCs), which antioxidants can help prevent (Chansiw et al., 2018). Anemia can result from physiological hemolysis, defined as the destruction of senescent RBCs after chemical and mechanical stresses that progressively damage them during their 120 days life span. This phenomenon increased hemoglobin catabolism, loss of membrane proteins, and altered ion transport. Macrophages remove old RBCs from the bloodstream (Badior and Casey, 2018). Moreover, anemia most commonly presents as pathological hemolysis due to alterations in the quantity

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and/or quality of proteins involved in maintaining the RBC membrane's properties, resulting in their increased and premature destruction. It may have a corpuscular or extracorporeal origin due to chronic, hereditary, or acquired destructions (Phillips and Henderson, 2018; Rai et al., 2020).

The polyunsaturated fatty acids of the membrane, the oxygen-rich environment, and the iron-rich hemoglobin make RBCs susceptible to oxidative damage. In RBC, hemoglobin is a major source of superoxide production. There is an electron transfer in the interaction between the heme iron and oxygen in oxygenated hemoglobin. The heme iron generally stays in the Fe(II) ferrous form when hemoglobin oxygenate. Alterations in this exchange, hemoglobin auto-oxidizes, resulting in methemoglobin and superoxide production (Fibach and Rachmilewitz, 2008). The denaturation of hemoglobin induced the formation of the Heinz body responsible for the rigidity of erythrocytes and thus their destruction (Drobatz et al., 2018). Furthermore, superoxide radical ($O_2^{\cdot}$) can dismutase spontaneously or enzymatically to hydrogen peroxide (H_2O_2), generating reactive hydroxyl radical ($OH^{\cdot}$). Consequently, ROS leads to the loss of membrane erythrocytes integrity by initiating lipid peroxidation (Fibach and Dana, 2019). Different aldehydes are formed as secondary oxidation products of lipid peroxidation, among them malondialdehyde (MDA) which leads to cross-links of phospholipids and proteins in the erythrocyte membrane. Indeed, MDA accumulation can affect anion transport and the functions of some enzymes. The processes alter membrane-related functions that ultimately lead to RBC hemolysis (Çimen, 2008; Ayala et al., 2014).

Rhamnus alaternus, commonly known as Buckthorn, belongs to the Rhamnaceae family. It is generally distributed throughout the Mediterranean countries. In North Africa, it grows in Algeria, Morocco, and northern Tunisia (Nekkaa et al., 2021). This plant is one species used in reforestation programs due to its ability to survive in xeric environments. In traditional medicine, the leaves are used as a purgative, laxative, and to treat jaundice (Moussi et al., 2015). In North African countries, the bark is used to cure various dermatological problems and treat diabetes (Nekkaa et al., 2021). *R. alaternus* crude extracts had powerful antimutagenic action as well as strong inhibition of xanthine oxidase. In addition, antioxidant, antiradical, antiproliferative, and cytotoxic properties were also demonstrated (Ben Ammar et al., 2007; Ben Ammar et al., 2008). Kosalec et al. (2013) reported that *R. alaternus* bark extracts contain anthraquinones and other phenols that act as multifunctional antioxidants with antimicrobial activity. While Benchiha et al. (2017) demonstrated that *R. alaternus* leaves extract has potent hepatoprotective properties against the xenobiotic carbon tetrachloride (CCl_4). According to our previous study, flavonoids obtained from *R. alaternus* leaves have an antihyperlipidemic effect on rats induced hyperlipidemic by Triton WR-1339 and on HepG2 cells culture (Tacherfiout et al., 2018).

Leaves and bark of *R. alaternus* are commonly used in an infusion to cure anemia in Algeria's Kabylia region. To our knowledge, no research has been conducted to determine whether *R. alaternus* possesses anti-hemolytic properties. Therefore, this work aimed to investigate the antihemolytic activity of *R. alaternus* leaves and bark extracts on human erythrocytes in a model of AAPH-induced hemolysis.

2. Materials and methods

2.1. Plant material

R. alaternus leaves and bark were harvested in May 2021 from Amizour, Bejaia, Algeria (GPS coordinates: 36°39'50'' North, 4°55'19'' East). They were separated, cleaned, rinsed, and dried for three days in the shade at room temperature before placing a 40 °C for 48 hours to enhance the drying process. Each part was ground in an electric grinder to have homogeneous powder (Ø = 125µm) kept in tightly sealed containers in the dark until extraction.

2.2. Preparation of the crude hydroalcoholic extracts

Fifty grams of each powder was mixed with 500 ml of an ethanol-water mixture (70/30: v/v) and kept under magnetic stirring at room temperature for 24 hours. Then, both mixtures were filtered three times through Whatman paper. The ethanol was completely evaporated using a rotary evaporator, and both extracts were placed in a laboratory oven at 40 °C for 48h to remove water traces. The extraction yield was calculated relative to the initial catch.

2.3. Phytochemical analysis

2.3.1. Determination of total polyphenol content

The total phenolic content of crude leaves and bark hydroalcoholic extracts was determined by Folin-Ciocalteu, which is reduced in the blue complex in the presence of phenols (Wolfe et al., 2003). A volume of 1.25 ml of Folin-Ciocalteu solution (0.1 N in distilled water) was mixed with 250 µl of each extract and then allowed to stand for 5 minutes. Afterward, 1 ml of 7.5% (w/v) sodium carbonate solution was added, and the absorbance was read at 720 nm in a spectrophotometer after 90 minutes of incubation in the dark. The concentration of total phenolic components in both extracts was expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g extract) using gallic acid as a calibration standard.

2.3.2. Determination of total flavonoid content

The total flavonoid content was determined using the aluminum trichloride ($AlCl_3$) method (Quettier-Deleu et al., 2000). Briefly, 1 ml of 2% aluminum trichloride solution (w/v) was mixed with the same volume of each extract. Absorption readings at 435 nm were taken after 15 minutes of incubation in the dark. Quercetin was used to prepare a standard curve, and the total flavonoid contents were expressed as milligram quercetin equivalent per gram of extract (mg QE/g).

2.3.3. Determination of flavone and flavonol content

The content of flavones and flavonols was determined according to the method described by Kosalec et al. (2004). 250 µl of the two hydroalcoholic extract solutions were mixed with 1.4 ml of distilled water, 750 µl of ethanol (95%), 50 µl of 10% $AlCl_3$ solution (w/v), and 50 µl of sodium acetate (1 M). The mixture was homogenized, and the absorbance was read at 438 nm after incubation for 15 minutes in the dark. In a calibration curve containing quercetin, the flavones and flavonol concentrations in the hydroalcoholic extracts were measured as milligram equivalents of quercetin per gram of extract (mg EQ/g).

2.3.4. Determination of condensed tannins content

The vanillin method described by Chaouche et al. (2020) was used to evaluate the content of condensed tannins in each extract. In brief, 1.5 ml of 4% (w/v) vanillin solution in methanol was mixed with 250 µl of each extract, and then 750 µl of sulfuric acid was added. The reaction mixture was allowed for 15 minutes, and the absorbance was recorded at 500 nm. A dilution series of catechin was prepared for a standard curve, and the results were expressed as milligram equivalent of catechin per gram of extract (mg EC/g).

2.4. Antioxidant activity

2.4.1. DPPH radical scavenging activity

The free radical scavenging activity of *R. alaternus* leaves and bark hydroalcoholic extracts was determined by the 2,2-diphényl-1-picrylhydrazyl (DPPH) method described by Athamena et al. (2010). A volume of 50 µl of DPPH solution (5 mM in methanol) was combined with 2.45 ml of each extract at different concentrations. The mixture was shaken and then incubated for 30 minutes in the dark, and the absorbance was then measured at 517 nm. Ascorbic acid was used as standard, and the percentages of DPPH radical scavenging activity were calculated using the following formula:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [Ac - As) / Ac] \times 100$$

Ac: Absorbance of control containing only DPPH

As: Absorbance of the sample containing DPPH and extracts or standard

2.4.2. ABTS radical scavenging activity

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical cation ($ABTS^{\cdot+}$) was produced by dissolving 7 mM ABTS in 2.45 mM potassium persulfate and keeping the solution in the dark for 16 hours. The ABTS stock solution was then diluted with ethanol to get an absorbance of 0.700 ± 0.02 at 734 nm. A volume of 950 µl of this diluted $ABTS^{\cdot+}$ solution is mixed with 50 µl of each extract at different doses. The absorbance reading was registered at 734 nm after 7 minutes of incubation in the dark (Re et al., 1999). Trolox was used as standard, and the following formula was used to determine the percentages of ABTS radical scavenging activity:

$$ABTS\ radical\ scavenging\ activity\ (\%) = [Ac - As) / Ac] \times 100$$

Ac: Absorbance of control containing only ABTS solution

As: Absorbance of the sample containing ABTS solution and extracts or standard

2.4.3. Reducing power

The reducing ability of the extracts was evaluated according to the method described by Kosalec et al. (2013). A volume of 500 µl of phosphate buffer (0.2 M, pH 6.6) and 500 µl of potassium ferricyanide 1% (w/v) were mixed with 250 µl of each extract. This mixture was kept at 50 °C in bath water for 20 minutes before cooling and adding 500 µl of 10% (w/v) trichloroacetic acid solution. After shaking, 500 µl was taken and combined with 500 µl of distilled water and 100 µl of 0.1% (w/v) ferric chloride solution. Absorbance was immediately read at 700 nm, and ascorbic acid was used to generate the calibration curve. Reducing power was expressed as milligram ascorbic acid equivalent per gram of extract (mg Asc E/g).

2.5. Antihemolytic activity on human erythrocytes

2.5.1. Preparation of erythrocyte suspension

Blood samples were obtained from healthy volunteers provided by the CTS (Centre de Transfusion Sanguine = Blood Transfusion Center, Bejaia, Algeria). Erythrocyte suspension was prepared according to the protocol described by Yang et al. (2017). Blood was centrifuged in heparinized tubes at 3000 rpm for 10 minutes. The supernatant was removed, and the pellet containing red blood cells was washed three times with phosphate buffer saline pH 7.4 (PBS) and centrifuged at 3000 rpm for 10 minutes. The erythrocyte pellet was resuspended at 10% hematocrit in PBS buffer and immediately used.

2.5.2. Hemolytic activity of extracts on human erythrocyte suspension

The hemolytic activity of leaves and bark extracts was assessed by mixing 250 µl of the freshly prepared erythrocyte suspension (10% hematocrit) with a volume of 500 µl of PBS (negative control), or 500 µl of each extract at different doses (25-1500 µg/ml). The samples were incubated for 4 hours at 37 °C in a water bath. The tubes were gently mixed every hour. A positive control was made by incubating the erythrocytes in 500 µl of distilled water simultaneously and under the same conditions (considered as 100% hemolysis). The volume of all tubes was then adjusted to 4.5 ml with PBS and centrifuged for 10 minutes at 2000 rpm. Hemolysis was determined by measuring absorbance at 540 nm, corresponding to hemoglobin release (Rafat et al., 2010). Hemolysis rates were calculated using the following formula:

$$Hemolysise\ rate\ (\%) = [(A_{extract} - A_{negative\ control} / A_{positive\ control}] \times 100$$

$A_{extract}$: Absorbance of hemoglobin at 540 nm in tubes treated with leaves and bark extracts

$A_{negative\ control}$: Absorbance of hemoglobin at 540 nm in tubes treated only with PBS

$A_{positive\ control}$: Absorbance of hemoglobin at 540 nm in tubes treated with distilled water

2.5.3. Optimization of hemolysis conditions of human erythrocytes by AAPH

AAPH was used in the present work to generate free radicals and oxidative stress, which could damage the RBC membrane and cause hemolysis. Briefly, 250 µl of erythrocyte suspension (10% hematocrit) was incubated at 37 °C in a water bath with 500 µl of AAPH dissolved in PBS at different final concentrations (100, 200, and 300 mM) for 4 hours. Every 60 minutes, the reaction mixture was gently stirred, and aliquots of the reaction mixture (100 µl) were removed and diluted with 500 µl of PBS. After centrifugation at 2000 rpm for 10 minutes, the absorbance of the supernatant was measured at 540 nm, and the percentages of hemolysis were then calculated as the previous formula (Phrueksanan et al., 2014).

2.5.4. Antihemolytic effects of the extract on AAPH induced hemolysis

The antihemolytic activity of the *R. alaternus* leaves and bark extracts was evaluated according to a previously published method (Yang et al., 2017). The erythrocyte suspension (hematocrit 10%) was pre-treated with different concentrations of the two extracts (50 - 200 µg/ml) for 30 minutes in a water bath at 37 °C. After that, a volume of 500 µl of an AAPH solution was added to have a final concentration of 200 mM, and the reaction mixture was incubated for 4 hours under the same conditions. Then, the volume of all tubes was adjusted to 4.5 ml with PBS and centrifuged at 2000 rpm for 10 minutes. The absorbance of the supernatant was read at 540 nm. Under the same conditions and simultaneously, two controls (negative and positive) were performed. Ascorbic acid was used as standard. The hemolysis inhibition percentages of extracts were calculated as follows.

$$Hemolysis\ inhibition\ (\%) = [(A_{AAPH} - A_{sample}) / A_{AAPH}] \times 100$$

A_{AAPH}: Absorbance at 540 nm in tubes treated only with 200 mM of AAPH

A_{sample}: Absorbance at 540 nm in tubes treated with 200 mM of AAPH and extracts or standard

2.6. Statistical analysis

All data values were expressed as mean ± standard deviation. All measurements were replicated three times. Statistical analyses were performed using Student's t-test or One-way ANOVA test followed by Tukey's post hoc test using Graphpad Prism 6.0 software. The results were considered statistically significant at $p < 0.05$. Determination of IC_{50} was performed using software Origin 9.5.

3. Results and discussion

3.1. Quantification of phytochemical compounds

The extraction yields of leaves and bark of *R. alaternus* using 70% ethanol were calculated concerning the initial dry matter. The results showed extraction yields of 19.8% for the leaf extract and 18.2% for the bark extract. The quantification of phytochemicals was assessed by colorimetric methods, and the results are reported in Table 1.

The results showed that leaves (LRA) were less rich in total phenolic contents than bark (BRA), with respective amounts of 80.22 ± 1.4 and 88.15 ± 1.83 mg GAE/g dry extract. These results were reported in mg gallic acid equivalent from the calibration curve equation (y = 0.0561x + 0.0093 with R^2=0.9916). There is a significant difference ($p < 0.05$) between the total polyphenol contents of the two extracts.

Table 1. Quantification of various phenolic compounds contents in hydroalcoholic leaves (LRA) and bark (BRA) extracts of *R. alaternus*

Phenolic compounds	LRA extract	BRA extract
Total polyphenols (mg GAE/g)	80.22 ± 1.4^a	88.15 ± 1.83^b
Total flavonoids (mg QE/g)	64.6 ± 2.6^a	39.87 ± 0.58^b
Flavones and flavonols (mg QE/g)	18.34 ± 1.65^a	10.08 ± 0.35^b
Condensed tannins (mg CE/g)	23.48 ± 0.25^a	36.24 ± 5.44^b

The mean and standard deviation (SD) are used to express the data ($n = 3$). The statistical analysis was determined using the Student's t-test. Values not sharing a common letter for the same type of compound were statistically different ($p < 0.05$).

In contrast, for the total flavonoid content of the two hydroalcoholic extracts, a higher level was obtained in leaves extract (LRA) (64.6 ± 2.6 mg QE/g dry extract) compared to the bark extract (BRA) (39.87 ± 0.58 mg QE/g dry extract). The results were expressed as mg quercetin equivalent based on a calibration curve that follows an equation y = 0.0428x - 0.028, with R^2 = 0.9895. From these findings, it was shown that there is a significant difference ($p < 0.05$) between the amount of total flavonoid content in the two extracts.

The flavone and flavonol content of leaves extract (LRA) was almost twice as high as that of the bark extract (BRA), with 18.34 ± 1.65 mg QE/g dry extract compared to 10.08 ± 0.35 mg EQ/g dry extract. The calibration curve was created using quercetin and yielded the following equation: y = 0.0712x + 0.0345 with R^2 = 0.9938. Statistical analysis of flavone and flavonol contents between the two extracts reveals a significant difference ($p < 0.05$).

Finally, the quantification of condensed tannins from the calibration curve performed using different concentrations of catechin (y = 0.0771x + 0.0528, with R^2 = 0.99633) showed that leaves extract (LRA) has a lower amount with a value of 23.48 ± 0.25 mg CE/g dry extract, than the bark extract (BRA) which had given a rate of 36.24 ± 5.44 mg CE/g dry extract. There is a significant difference ($p < 0.05$) in the condensed tannins contents between the two extracts.

3.2. Antioxidant activities

3.2.1. DPPH free radical scavenging activity

Leaves and bark extracts of *R. alaternus* were evaluated for their ability to quench DPPH radicals. As shown in Figure 1, both extracts' DPPH radical scavenging activity is dose-dependent. In fact, at the highest concentration tested (125 µg/ml), leaves (LRA) and bark (BRA) extracts exhibited potent scavenging activities with percentages of 73.18 ± 0.65% and 81.21 ± 1.5%, respectively. The DPPH free radical scavenging activity of the two extracts was statistically different ($p < 0.05$). As can be seen in Table 2, the bark extract showed the highest antioxidant activity with an IC_{50} = 69.23 ± 2.14 µg/ml, compared to 86.59 ± 2 µg/ml for leaves extract. Ascorbic acid used as standard gave IC_{50} = 1.04 ± 0.10 µg/ml.

3.2.2. ABTS free radical scavenging activity

The antioxidant ability of both extracts toward the ABTS radical demonstrates that leaves and bark hydroalcoholic extracts are effective even at low doses (Figure 2). A considerable scavenging effect of the ABTS radical was observed at 22.5 µg/ml, with percentages of 70 ± 1.39% for leaves extract and 75.88 ± 0.72% for the bark extract. The ABTS radical scavenging activities of the two extracts were significantly different ($p < 0.05$), except at the 12.5 µg/ml concentration, where the difference was not significant. Moreover, this high scavenging activity of the ABTS radical of both

extracts is illustrated by the IC_{50} values obtained; 12.49 ± 0.29 µg/ml for leaves extract and 12.83 ± 0.13 µg/ml for bark extract (Table 2). Trolox used as a standard in this test gave an IC_{50} of 8.38 ± 0.08 µg/ml.

3.2.3. Reducing power

Ascorbic acid was used as the standard to create the calibration curve following an equation: y = 0.044x + 0.3494 with R^2 = 0.9984. Thus, ascorbic acid equivalents in mg/g extract were used to represent the reducing capacity of *R. alaternus* leaves and bark extracts. Table 2 showed that both extracts have a good reducing capacity, with a slight advantage for the bark extract, which gave a reducing power of 194.97 ± 1.46 mg Asc E/g, compared to leaves extract (157.09 ± 5.53 mg Asc E/g). There was a significant difference between the reducing powers of the two extracts (p < 0.05).

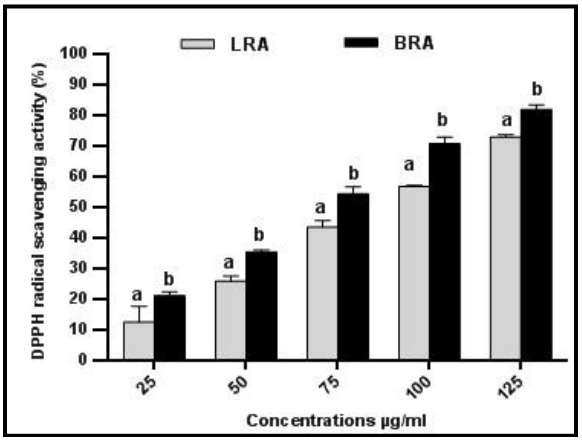

Figure 1. DPPH radical scavenging activity of leaves (LRA) and bark (BRA) extracts (25-125 µg/ml) of *R. alaternus*
Values are mean ± SD (n = 3). The Student's t-test was used to determine the statistical significance of the data. Values not sharing a common letter for the same concentration were statistically different (p < 0.05).

Table 2. Reducing capacity of *R. alaternus* leaves (LRA) and bark (BRA) hydroalcoholic extracts and IC_{50} values for DPPH and ABTS tests

Extract	Reducing power (mg Asc E/g)	DPPH (µg/ml)	ABTS (µg/ml)
LRA	157.09 ± 5.53^a	86.59 ± 2.00^a	12.49 ± 0.29^a
BRA	194.97 ± 1.46^b	69.23 ± 2.14^b	12.83 ± 0.13^a
Ascorbic acid	-	1.04 ± 0.10^c	-
Trolox	-	-	8.38 ± 0.08^b

The mean and standard deviation (SD) are used to express the data (n = 3). The statistical analysis was determined using the Student's t-test. Values not sharing a common letter for the same type of compound were statistically different (p < 0.05).

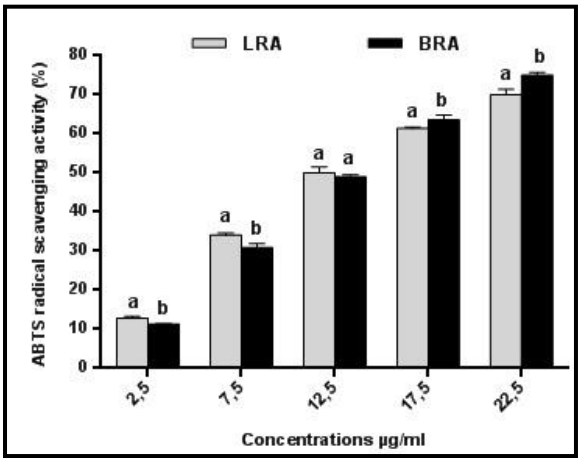

Figure 2. ABTS radical scavenging activity of leaves (LRA) and bark (BRA) extracts (2.5-22.5 µg/ml) of *R. alaternus*
Values are mean ± SD (n = 3). The Student's t-test was used to determine the statistical significance of the data. Values not sharing a common letter for the same concentration were statistically different (p < 0.05).

3.3. Cytotoxicity of R. alaternus leaves and bark extracts on human erythrocytes

The hemolytic activity of different concentrations of leaves and bark extracts was tested on human erythrocytes (Figure 3). Statistical analysis showed a significant difference (p < 0.05) between hemolysis rates induced by the two extracts and complete hemolysis induced by H_2O. The results demonstrate that leaves extract (LRA) induced a very low cytotoxicity potential at concentrations ranging from 25 to 1000 µg/ml. Indeed, hemolysis rates were 1.06 ± 0.98% at 25µg/ml and 0.71 ± 0.75% at 1000 µg/ml. However, at a 1500 µg/ml concentration, the hemolysis rate rises to roughly 5%. On the other hand, hemolysis rates for the bark extract (BRA) were very low at doses ranging from 25 µg/ml (0.09 ±

0.28%) to 500 µg/ml (3.18 ± 0.24%), but increased to 5.5 ± 1.18% at 750 µg/ml, 7.65 ± 0.81% at 1000 µg/ml and 14.9 ± 0.18% at 1500 µg/ml.

3.4. Optimization of hemolysis conditions of human erythrocytes by AAPH

Human erythrocytes were incubated at 37 °C as a 10% hematocrit suspension with AAPH at different final concentrations of 100, 200, and 300 mM. Hemolysis rates were then monitored hourly by measurement at 540 nm of hemoglobin released into the incubation medium. Figure 4 showed that AAPH induced hemolysis in a time and concentration-dependent manner. The onset of hemolysis occurred after 1 h of incubation (16.26 ± 2.75%) for the 300 mM concentration, after 2 h of incubation (31.41 ± 1.52%) for the 200 mM concentration, and after 3 h of incubation (11.42 ± 7.02%) for the 100 mM concentration. In these experimental conditions, the hemolysis was markedly induced after 4 h of incubation at 200 mM and 300 mM with respective percentages of 45 ± 1.25% and 49.24 ± 4.15%. Therefore, AAPH at 200 mM and the incubation period of 4 h were selected for hemolysis conditions in this study.

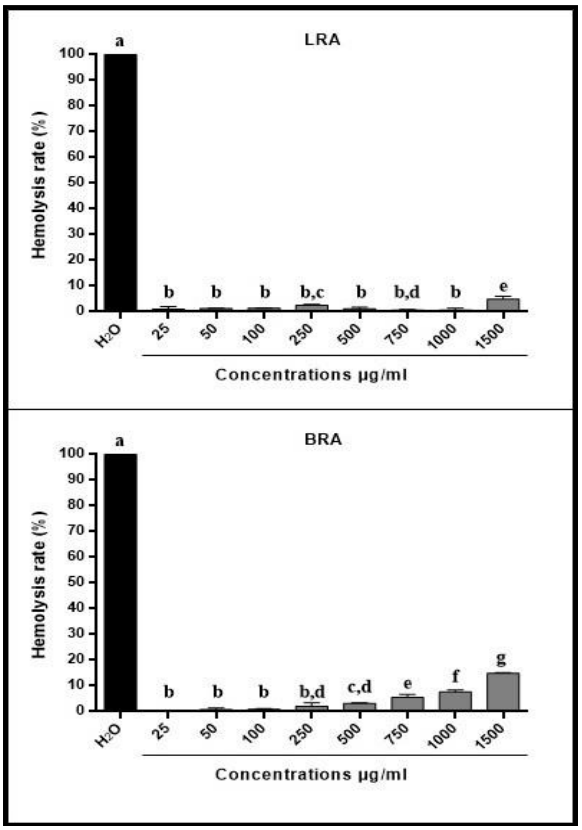

Figure 3. Hemolysis rates induced on human red blood cells (10% hematocrit) by various doses of *R. alaternus* leaves and bark extracts (25–1500 µg/ml)

Values are expressed as mean ± standard deviation (n = 3). Differences between means were statistically tested by one-way ANOVA test, followed by Tukey's post hoc test. Values not sharing a common letter were statistically different ($p < 0.05$).

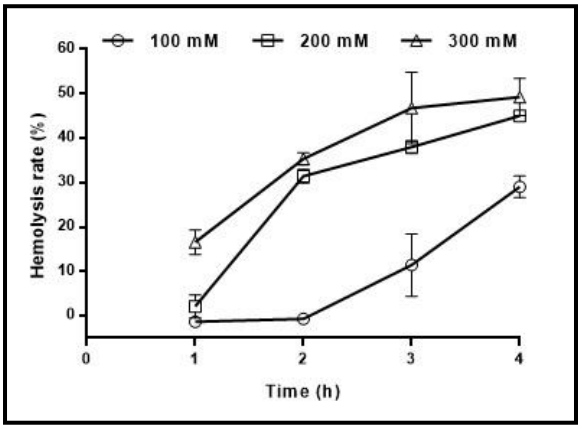

Figure 4. Time-dependent variations in hemolysis rates induced by different final concentrations of AAPH (100, 200, and 300 mM) on human red blood cells

Results are expressed as mean ± standard deviation (n = 3).

3.5. Antihemolytic activity of leaves and bark extracts against AAPH-induced hemolysis on human erythrocytes

The antihemolytic activity of LRA and BRA was assessed by measuring the hemolysis degree induced in human erythrocytes by AAPH-generated free radicals. Pretreatment of human red blood cells by different doses (50-200 µg/ml) of the two extracts of *R. alaternus* significantly attenuated the AAPH-induced hemolysis concentration-dependent (Figure 5). The results clearly show that the BRA was more efficient than LRA extract in reducing AAPH-induced hemolysis, especially at concentrations of 150 and 200 µg/ml. Indeed, the hemolysis percentages inhibition for the bark extract were 91.91 ± 1.77% and 99.4 ± 1.17% (almost 100% inhibition) at the concentrations of 150 and 200 µg/ml, respectively, against 52.69 ± 1.75% and 76.26 ± 12.02% for leaves extract at the same concentrations. Whereas 100 µg/ml of ascorbic acid was used as a standard, giving a percentage inhibition of 34.75 ± 3.7 % (Table 3). This strongest antihemolytic activity of the bark extract compared to leaves extract is illustrated by the IC_{50} values obtained (concentrations inducing 50% inhibition of AAPH-induced hemolysis). The bark extract had an IC_{50} = 106.70 ± 1.48 µg/ml, while leaves extract had an IC_{50} = 148.64 ± 7.04 µg/ml. Data statistical analysis revealed no significant variations in hemolysis rates between leaves extract concentrations of 50, 100 µg/ml, and ascorbic acid. However, in the presence of different doses of bark extract, ascorbic acid, APPH, and positive control (100% hemolysis), there is a statistically significant difference in hemolysis rates (p < 0.05).

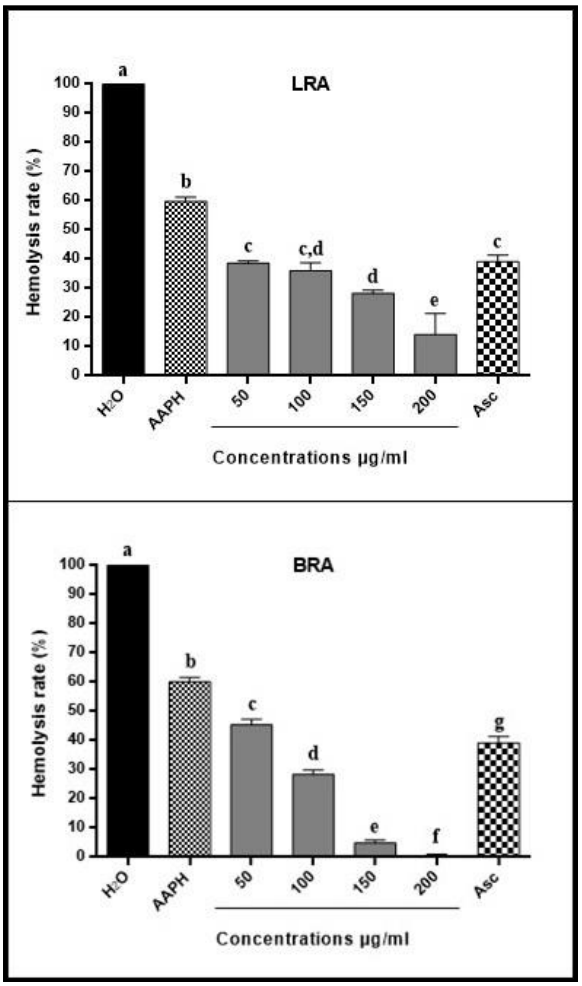

Figure 5. Antihemolytic effect of leaves (LRA) and bark (BRA) extracts from *R. alaternus* at different concentrations against AAPH-induced hemolysis on human erythrocytes

Values are expressed as mean ± standard deviation (n = 3). Differences between means were statistically tested by one-way ANOVA test, followed by Tukey's post hoc test. Values not sharing a common letter were statistically different (p < 0.05).

Table 3. Inhibition rates and IC_{50} values of AAPH induced hemolysis after pretreatment of human erythrocytes with different concentrations of leaves (LRA) and bark (BRA) extracts of *R. alaternus*

Concentration (µg/ml)	LRA hemolysis inhibition (%)	BRA hemolysis inhibition (%)
50	35.4 ± 1.32^{a}	24.49 ± 3.11^{b}
100	39.76 ± 4.59^{a}	52.78 ± 2.51^{b}
150	52.69 ± 1.75^{a}	91.91 ± 1.77^{b}
200	76.26 ± 12.02^{a}	99.4 ± 1.17^{b}
Ascorbic acid 100 µg/ml	34.75 ± 3.7	
IC_{50}	148.64 ± 7.04^{a}	106.70 ± 1.48^{b}

Data are expressed as mean ± standard deviation (n = 3). IC_{50}: Concentration of test substances reducing the cell viability by 50%. The statistical analysis was determined using the Student's t-test. Values not sharing a common letter for the same concentration were statistically different (p < 0.05).

Plants include various bioactive natural compounds used in traditional medicine for their healing power (Kumar et al., 2011). In this context, the purpose of this study was to assess the phytochemicals content, antihemolytic and antioxidant activities of *R. alaternus* hydroalcoholic leaves and bark extracts. Our findings demonstrate that different extraction yields and variable contents of various phenolic compounds were obtained compared to earlier investigations. The harvesting period, geographical location, and meteorological and experimental circumstances all play a role in these variances (Bouchenak et al., 2020). Furthermore, various parameters such as pH, temperature, maceration time, solid/liquid ratio, powder granulometry, the chemical structure of the compounds, and solvent nature might influence the extraction. Indeed, for the extraction of usually polar phenolic compounds, various polar solvents such as ethanol, methanol, and acetone are frequently utilized (Benmeziane et al., 2014; Bouchenak et al., 2020). In addition, the solvent can influence the permeability of plant cells by chemical and biophysical changes, and its efficiency is mostly determined by its capacity to dissolve phenolic groups (Oreopoulou et al., 2019). In this study ethanol was used because it solubilizes medium polar phenolic compounds, and the addition of water to the extraction system improves the yield of glycosylated and highly polymerized phenolic compounds (Nga et al., 2019). It is also preferred since it is less toxic and environmentally friendly (Cheok et al., 2014).

Results showed that bark extract of *R. alaternus* contains more total polyphenols than leaf extract. This may be due to the part of the plant studied, as phenolic compounds are distributed differently throughout the different plant organs (Bouchenak et al., 2020). Ben Ammar et al. (2007) and Chaouche et al. (2020) reported that total polyphenol levels respectively of 138 mg GAE/g extract for methanolic extract and 1.68 mg GAE/g extract for methanol/acetone extract of *R. alaternus* leaves. However, total polyphenol contents reported by Chaouche et al. (2020) for methanol/acetone extract and Kosalec et al. (2013) for the methanolic extract from the bark of *R. alaternus* were ranged between 0.64 mg EAG/g extract and 38.4 mg EAG/g extract, respectively. These variations can be explained by the origin and harvesting period of the plant, the type of solvent used, drying duration as well as the extraction method (Benchaachoua et al., 2018; Chaouche et al., 2020). This also could be due to the Folin-Ciocalteu reagent's limitation, which provides a crude assessment of all phenolic chemicals and can be reduced by other molecules such as reducing sugars and proteins (Bessada et al., 2015).

Flavonoids are a class of polyphenols that are widely distributed throughout the plant kingdom and in all parts of higher plants (Benchaachoua et al., 2018). They have a specific structure that includes the positions of hydroxyl and carbonyl groups as well as a double bond, which results in flavone and flavonol derivatives (Ahmed et al., 2019). In this study, the contents of total flavonoids, flavones, and flavonols were predominantly higher in the leaves extract than in the bark extract. These results are confirmed by Ben Ammar et al. (2007) and Kosalec et al. (2013), who had found flavonoid concentrations of 283 mg QE/g and 33.6 mg QE/g, respectively, in methanolic leaves and bark extracts. This can be explained by the difference in the distribution of flavonoids during plant development and because flavones and flavonols are mainly concentrated in the plant's leaves due to their high exposure to sunlight (Benchaachoua et al., 2018; Ahmed et al., 2019).

Moreover, both leaves and bark extracts contain a significant amount of condensed tannins, with the bark extract having a higher level than the leaves (36.24 ± 5.44 and 23.48 ± 0.25 mg CE/g extract, respectively). However, Ben Ammar et al. (2007) found 736 mg TAE/g extract for the methanolic leaves extract, and Chaouche et al. (2020) found 3 mg CE/g extract for the methanol/acetone bark extract. This discrepancy could be related to the structure of condensed tannins and their solubility, which is influenced by several factors, including the plant's developmental region and the solvent utilized (Elgailani and Ishak, 2016; Chaouche et al., 2020).

Three methods were used to assess the antioxidant activities of the two extracts: DPPH and ABTS radical scavenging activities, as well as reducing power. For the first two procedures, the data are expressed in IC_{50}, the concentration of extracts required to quench 50% of the DPPH and ABTS radicals, and in equivalent ascorbic acid per gram of extract for the reducing power method. DPPH assay showed that bark extract (IC_{50} = 69.23 ± 2.14 µg/ml) had a higher ability to quench the DPPH radical than leaves extract (IC_{50} = 86.59 ± 2.00 µg/ml). In comparison, previous studies showed higher DPPH radical scavenging activity with an IC_{50} = 58 ± 0.007 µg/ml for the ethanolic leaves extract (Zeouk et al., 2020) and lower activity with an IC_{50} = 78.7 ± 3.16 µg/ml for the methanolic bark extract (Kosalec et al., 2013). The difference in DPPH radical quenching between the two extracts could be due to the nature of the phenolic compounds contained in each extract, which can interact in various ways to reduce free radicals (Abate, 2019). The diversity of our results compared to those reported later could be due to the nature of the solvent used and the solvent/water ratio, which significantly impact the total antioxidant activity of the extracts and favor the extraction of potent antioxidants (Benchaachoua et al., 2018). Furthermore, leaves and bark extracts were found to have similar powers to quench the ABTS free radical (IC_{50} = 12.49 ± 0.29 and 12.83 ± 0.13 µg/ml for leaves and bark extracts, respectively). This can be explained by the low sensitivity of the ABTS assay, which cannot distinguish between various bioactive compounds found in plants (Chaves et al., 2020). The difference in results between the DPPH and ABTS assays can be explained by the variety of antioxidant chemicals found in both extracts, which respond differently depending on the radical present. One downside of ABTS is that the produced free radicals are not very stable (Shah and Modi, 2015).

The bark extract (194.97 ± 1.46 mg Asc E/g) was shown to be more powerful than the leaves extract (157.09 ± 5.53 mg Asc E/g) in the reducing power test. The amount of electron-donating phenolic compounds capable of inhibiting the free radical chain reaction may be proportionate to the extracts' reducing capacity (Kosalec et al., 2013). The reducing ability of bioactive compounds is also influenced by their chemical structures, such as the number and placement of hydroxyl groups concerning electron-withdrawing carboxyl groups (Enneb et al., 2015).

The erythrocytes are frequently used as a biological model to assess the antihemolytic and antioxidant potential of different compounds and plant extracts because of their high sensitivity to oxidative stress due to their membrane lipids rich in polyunsaturated fatty acids. Indeed, they are the first target of free radical attack due to their potential as a producer of reactive oxygen species (ROS), as well as the redox reactions of hemoglobin associated with oxygen transfer (Hmidani et al., 2021; Derouich et al., 2020). Many bioactive molecules present in plants can affect biological membranes due to their cytotoxicity (Ramchoun et al., 2015). In the present case, the hemolytic activity of *R. alaternus* leaves and bark extracts was evaluated on human red blood cells. Leaves extract showed no cytotoxic effect at concentrations ranging from 25 to 1000 µg/ml. In contrast, the bark extract caused hemolysis at concentrations greater than 500 µg/ml. At a 1500 µg/ml concentration, this hemolysis rate was around 15%. Our findings suggest that the two

extracts did not have cytotoxic effects at low doses on human red blood cells.

In this study, an assessment was also performed on the ability of leaves and bark extracts to protect red blood cells from damage caused by AAPH-induced hemolysis. Indeed, the AAPH-induced hemolysis model is widely used to estimate plant extracts' antioxidant and antihemolytic capacity. At 37 °C, AAPH generates a low but constant flux of peroxyl radicals, producing free radicals that cause erythrocytes, membrane lipids, and proteins to undergo chain oxidation. Thus, the disruption of the erythrocytes membrane caused by free radical excess ultimately leads to hemolysis or death of healthy erythrocyte cells (Yuan et al., 2005; Ramchoun et al., 2015). The significant increase in hemolysis rate in erythrocytes treated only with 200 mM APPH and incubated for 4 hours can be explained because continuous exposure of erythrocytes to APPH resulted in maximal utilization of endogenous antioxidants (Yang et al., 2017).

The anti-hemolytic activity demonstrated that treating erythrocytes with *R. alaternus* leaves and bark extracts enhanced their resistance to AAPH damage in a dose-dependent manner. Bark extract (BRA) showed a better protective effect on the erythrocyte membrane than leaves extract (LRA). In fact, at a 200 µg/ml concentration, the bark extract almost completely inhibited AAPH-induced hemolysis with an inhibition rate of 99.41 ± 1.17%, which was much higher than the leaves extract inhibition rate of 76.26 ± 12.03%. This antihemolytic activity is probably due to the various phenolic compounds in both extracts, known as phenolic hydrogen atom donors. They help stabilize free radicals through their antioxidant activity, increasing erythrocytes' oxidative stress resistance. Moreover, the phenolic components present in both extracts could donate one or more electrons to neutralize the AAPH radical while inhibiting hemolysis at the same time. In addition, polyphenols have also been shown to have the ability to interact with the hydrophilic section of the lipid membrane, causing changes in the packing arrangement of the polar heads of the lipids (Balderrama-Carmona et al., 2020).

Furthermore, several previous studies on *R. alaternus* leaves and bark extracts revealed the presence of multiple compounds, mainly glycosylated and non-glycosylated flavonoids such as kaempferol, quercetin, kaempferol hexoside, kaempferol 3-*O*-acetyl-rhamnoside, rhamnetin hexoside, and rhamnocitrin hexoside, etc. (Ben Ammar et al., 2009; Boussahel et al., 2015; Moussi et al., 2015; Tacherfiout et al., 2018). These various flavonoids, as well as other polyphenols found in both extracts, may protect erythrocyte cell membranes by interacting with membrane phospholipids, which protect them from lipid deterioration (Derouich et al., 2020). Flavonoids also promote Van der Waals contacts within the lipid bilayer and may be a source of membrane stability, protecting erythrocytes from free radical damage (Ramchoun et al., 2015).

Kaempferol, one of the flavonoids found in *R. alaternus*, has already demonstrated anti-hemolytic action. Indeed, according to Liao et al. (2016), the hydroxyl group at the C3 position plays a critical role in flavonoids' high antioxidant action via controlling heme oxygenase. In addition, kaempferol protects erythrocyte membrane architecture against AAPH-induced structural damage, lowers ROS-induced hemolysis in a dose-dependent manner, and has excellent anti-lipid peroxidation.

4. Conclusions

In summary, *R. alaternus* has a variety of phenolic compounds with good antioxidant properties. The results of this study appear to back up the traditional usage of *R. alaternus* to treat hemolytic anemia. We demonstrated that *R. alaternus* attenuates AAPH-induced hemolysis on human red blood cells for the first time. Leaves and hydroalcoholic bark extracts significantly reduced AAPH-induced hemolysis. *R. alaternus*' antihemolytic action could be attributed to its antioxidant status, which could protect erythrocytes from oxidative damage and hemolysis. *R. alaternus*' bioactive potential could be highly useful in preventing and treating hemolytic anemias and disorders involving excessive oxidative stress generation.

Acknowledgments

None.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

Sarah Kherbachi: Data curation, Investigation, Writing - original draft
Meriem Kheniche: Data curation, Investigation
Mustapha Tacherfiout: Conceptualization, Methodology, Supervision, Writing-reviewing & editing

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Supplementary File

None.

References

Abate, L., 2019. Bioactivity profiling of four traditional medicinal plants leave extracts native to Alemsaga Forest, Ethiopia. *Chemistry International*, 5(4), 281-290.

Ahmed, J., Salim, K.A., Lim, L.B., Jama, A.M., 2019. Evaluation of Antioxidant Activity and Phytochemical Screening of Leaves, Barks, Stems and Fruits of *Alphitonia philippinensis* (Rhamnaceae) From Brunei Darussalam. *Pharmacognosy Journal*, 11(5), 951-961.

Athamena, S., Chalghem, I., Kassah-Laouar, A., Laroui, S., Khebri, S., 2010. Activité antioxydante et antimicrobienne d'extraits de *Cuminum cyminum* L. *Lebanese Science Journal*, 11(1), 69-81.

Ayala, A., Muñoz, M.F., Argüelles, S., 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*, 2014, 360438.

Badior, K.E., Casey, J.R., 2018. Molecular mechanism for the red blood cell senescence clock. *International Union of Biochemistry and Molecular Biology Life*, 70(1), 32-40.

Balderrama-Carmona, A.P., Silva-Beltrán, N.P., Gálvez-Ruiz, J.C., Ruíz-Cruz, S., Chaidez-Quiroz, C., Morán-Palacio, E.F., 2020. Antiviral, antioxidant, and antihemolytic effect of *Annona muricata* L. leaves extracts. *Plants*, 9(12), 1650.

Ben Ammar, R., Bouhlel, I., Valenti, K., Sghaier, M.B., Kilani, S., Mariotte, A.M., Chekir-Ghedira, L., 2007. Transcriptional response of genes involved in cell defense system in human cells stressed by H_2O_2 and pre-treated with (Tunisian) *Rhamnus alaternus* extracts: Combination with polyphenolic compounds and classic *in vitro* assays. *Chemico-Biological Interactions*, 168(3), 171-183.

Ben Ammar, R., Sghaier, M.B., Boubaker, J., Bhouri, W., Naffeti, A., Skandrani, I., Chekir-Ghedira, L., 2008. Antioxidant activity and inhibition of aflatoxin B1-, nifuroxazide-, and sodium azide-induced mutagenicity by extracts from *Rhamnus alaternus* L. *Chemico-Biological Interactions*, 174(1), 1-10.

Ben Ammar, R., Bhouri, W., Sghaier, M.B., Boubaker, J., Skandrani, I., Neffati, A., Ghedira, K., 2009. Antioxidant and free radical-scavenging properties of three flavonoids isolated from the leaves of *Rhamnus alaternus* L.(Rhamnaceae): A structure-activity relationship study. *Food Chemistry*, 116(1), 258-264.

Benchaachoua, A., Bessam, H.M., Saidi, I., Bel-abbes, S., 2018. Effects of different extraction methods and solvents on the phenolic composition and antioxidant activity

of *Silybum marianum* leaves extracts. *International Journal of Medical Science and Clinical Invention*, 5(3), 3641-3647.
Benchiha, W., Mahroug, S., Aoued, L., Bouterfas, K., 2017. Activité antihépatotoxique des extraits des feuilles de *Rhamnus alaternus* L.(Rhamnaceae). *Phytothérapie*, 15(1), 10-15.
Benmeziane, F., Djamai, R., Cadot, Y., Seridi, R., 2014. Optimization of extraction parameters of phenolic compounds from Algerian fresh table grapes,(*Vitis vinifera*). *International Food Research Journal*, 21(3), 1061-1065.
Bessada, S.M., Barreira, J.C., Oliveira, M.B.P., 2015. Asteraceae species with most prominent bioactivity and their potential applications: A review. *Industrial Crops and Products*, 76, 604-615.
Bouchenak, O., Yahiaoui, K., Benhabyles, N., Laoufi, R., Toubal, S., El Haddad, D., Arab, K., 2020. Criblage phytochimique et évaluation du pouvoir antioxydant des feuilles de myrtus communis L. et *Rhamnus alaternus* L. *Revue Agrobiologia*, 10, 1749-1761.
Boussahel, S., Speciale, A., Dahamna, S., Amar, Y., Bonaccorsi, I., Cacciola, F., Cristani, M., 2015. Flavonoid profile, antioxidant and cytotoxic activity of different extracts from Algerian *Rhamnus alaternus* L. bark. *Pharmacognosy Magazine*, 11(Suppl 1), S102-S109.
Chansiw, N., Paradee, N., Chotinantakul, K., Srichairattanakool, S., 2018. Anti-hemolytic, antibacterial and anti-cancer activities of methanolic extracts from leaves and stems of *Polygonum odoratum*. *Asian Pacific Journal of Tropical Biomedicine*, 8(12), 580.
Chaouche, T.M., Haddouchi, F., Boudjemai, O., Ghellai, I., 2020. Antioxidant and hemolytic activity of *Ziziphus jujuba* Mill and *Rhamnus alaternus* L (Rhamnaceae) extracts from Algeria. Activité antioxydante et hémolytique des extraits de *Ziziphus jujuba* Mill et *Rhamnus alaternus* (Rhamnaceae) d'Algérie. *Bulletin de la Société Royale des Sciences de Liège*, 89, 1-14.
Chaves, N., Santiago, A., Alías, J.C., 2020. Quantification of the antioxidant activity of plant extracts: Analysis of sensitivity and hierarchization based on the method used. *Antioxidants*, 9(1), 76.
Cheok, C.Y., Salman, H.A.K., Sulaiman, R., 2014. Extraction and quantification of saponins: A review. *Food Research International*, 59, 16-40.
Çimen, M.B., 2008. Free radical metabolism in human erythrocytes. *Clinica Chimica Acta*, 390(1-2), 1-11.
Derouich, M., Bouhlali, E.D.T., Bammou, M., Hmidani, A., Sellam, K., Alem, C., 2020. Bioactive compounds and antioxidant, antiperoxidative, and antihemolytic properties investigation of three apiaceae species grown in the southeast of Morocco. *Scientifica*, 2020, 3971041.
Drobatz, K.J., Hopper, K., Rozanski, E.A., Silverstein, D.C., 2018. Hemolytic anemia. In Wiley Blackwell (Eds), Textbook of Small Animal Emergency Medicine (pp. 419-443). John Wiley and Sons.
Elgailani, I.E.H., Ishak, C.Y., 2016. Methods for extraction and characterization of tannins from some *Acacia* species of Sudan. Pakis*tan Journal of Analytical & Environmental Chemistry*, 17(1), 43-49.
Enneb, H., Belkadhi, A., Cheour, F., Ferchichi, A., 2015. Comparaison des composés phénoliques et du pouvoir antioxydant de la plante de henné (*Lawsonia inermis* L.). *Journal of New Sciences*, 20, 788-793.
Fibach, E., Dana, M., 2019. Red blood cells as redox modulators in hemolytic anemia. In Tombak Anil (Eds). Erythrocyte (pp. 49-63). IntechOpen publishers.
Fibach, E., Rachmilewitz, E., 2008. The role of oxidative stress in hemolytic anemia. *Current Molecular Medicine*, 8(7), 609-619.
Hmidani, A., Ajebli, M., Khouya, T., Benlyas, M., Alem, C., 2021. *In vitro* investigation of antioxidant and antihemolytic activities of three Lamiaceae species from Morocco. *Beni-Seuf University Journal of Basic Applied Sciences*, 10, 1-8.
Kosalec, I., Bakmaz, M., Pepeljnjak, S., Vladimir-Knezevic, S., 2004. Quantitative analysis of the flavonoids in raw propolis from northern Croatia. *Acta Pharmaceutica*, 54(1), 65-72.
Kosalec, I., Kremer, D., Locatelli, M., Epifano, F., Genovese, S., Carlucci, G., Končić, M.Z., 2013. Anthraquinone profile, antioxidant and antimicrobial activity of bark extracts of *Rhamnus alaternus*, *R. fallax*, *R. intermedia* and *R. pumila*. *Food Chemistry*, 136(2), 335-341.
Kumar, G., Karthik, L., Rao, K.V.B., 2011. Hemolytic activity of Indian medicinal plants towards human erythrocytes: an *in vitro* study. *Elixir Applied Botany*, 40, 5534-5537.
Liao, W., Chen, L., Ma, X., Jiao, R., Li, X., Wang, Y., 2016. Protective effects of kaempferol against reactive oxygen species-induced hemolysis and its antiproliferative activity on human cancer cells. *European Journal of Medicinal Chemistry*, 114, 24-32.
Moussi, K., Nayak, B., Perkins, L.B., Dahmoune, F., Madani, K., Chibane, M., 2015. HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves extract of *Rhamnus alaternus* L. *Industrial Crops and Products*, 74, 858-866.
Nekkaa, A., Benaissa, A., Mutelet, F., Canabady-Rochelle, L., 2021. *Rhamnus alaternus* Plant: Extraction of Bioactive Fractions and Evaluation of Their Pharmacological and Phytochemical Properties. *Antioxidants*, 10(2), 300.
Nga, E.N., Nchinda, G., Mahi, L.L., Figuei, P.B., Mvondo, J.M., Sagnia, B., Adiogo, D., 2019. Phytochemical screening and evaluation of antioxidant power of hydro-ethanolic and aqueous leaves extracts of *Annona muricata* Linn (soursop). *Health Sciences and Disease*, 20(1), 29-33.
Oreopoulou, A., Tsimogiannis, D., Oreopoulou, V., 2019. Extraction of polyphenols from aromatic and medicinal plants: An overview of the methods and the effect of extraction parameters. In Ronald Ross Watson (Eds). Polyphenols in plants (pp. 243-259). Academic Press publishers.
Phillips, J., Henderson, A.C., 2018. Hemolytic anemia: evaluation and differential diagnosis. *American Family Physician*, 98(6), 354-361.
Phrueksanan, W., Yibchok-anun, S., Adisakwattana, S., 2014. Protection of *Clitoria ternatea* flower petal extract against free radical-induced hemolysis and oxidative damage in canine erythrocytes. *Research in Veterinary Science*, 97(2), 357-363.
Quettier-Deleu, C., Gressier, B., Vasseur, J., Dine, T., Brunet, C., Luyckx, M., Trotin, F., 2000. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *Journal of Ethnopharmacology*, 72(1-2), 35-42.
Rafat, A., Philip, K., Muni, S., 2010. Antioxidant potential and content of phenolic compounds in ethanolic extracts of selected parts of *Andrographis paniculata*. *Journal of Medicinal Plants Research*, 4(3), 197-202.
Rai, M., Ali, M.U., Geller, C., 2020. Hemolytic Anemia: Sneaky Cause, Leaky Valve. *Cureus*, 12(5), 1-9.
Ramchoun, M., Sellam, K., Harnafi, H., Alem, C., Benlyas, M., Khallouki, F., Amrani, S., 2015. Investigation of antioxidant and antihemolytic properties of *Thymus satureioides* collected from Tafilalet Region, south-east of Morocco. *Asian Pacific Journal of Tropical Biomedicine*, 5(2), 93-100.
Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26(9-10), 1231-1237.
Shah, P., Modi, H.A., 2015. Comparative study of DPPH, ABTS and FRAP assays for determination of antioxidant activity. *International Journal of Research in Applied Science and Engineering Technology*, 3(6), 636-641.
Tacherfiout, M., Petrov, P.D., Mattonai, M., Ribechini, E., Ribot, J., Bonet, M.L., Khettal, B., 2018. Antihyperlipidemic effect of a *Rhamnus alaternus* leaf extract in Triton-induced hyperlipidemic rats and human HepG2 cells. *Biomedicine & Pharmacotherapy*, 101, 501-509.
Wolfe, K., Wu, X., Liu, R.H., 2003. Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*, 51(3), 609-614.
Yang, H.L., Korivi, M., Lin, M.K., Chang, H.C.W., Wu, C.R., Lee, M.S., Hseu, Y.C., 2017. Antihemolytic and antioxidant properties of pearl powder against 2, 2'-azobis (2-amidinopropane) dihydrochloride-induced hemolysis and oxidative damage to erythrocyte membrane lipids and proteins. *Journal of Food and Drug Analysis*, 25(4), 898-907.
Yuan, X., Wang, J., Yao, H., 2005. Antioxidant activity of feruloylated oligosaccharides from wheat bran. *Food Chemistry*, 90(4), 759-764.
Zeouk, I., Ouedrhiri, W., Jiménez, I.A., Lorenzo-Morales, J., Bazzocchi, I.L., Bekhti, K., 2020. Intra-combined antioxidant activity and chemical characterization of three fractions from *Rhamnus alaternus* extract: Mixture design. *Industrial Crops and Products*, 144, 112054.

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RESEARCH ARTICLE

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A comparative assessment of antifungal activity of essential oils of five medicinal plants from Tunisia

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ABSTRACT

The leaf essential oil yields of clementine, cypress, rosemary, tea, and thyme were 0.22, 0.87, 1.46, 1.20, and 0.72%, respectively, based on the dry weight of the plant material. The leaf essential oils of rosemary, tea, and thyme contained the highest levels of oxygenated monoterpenes (60.14-91.70%). Rosemary and tea leaf essential oils were rich in 1,8-cineole (49.98% and 57.55%, respectively), and they have potent antifungal activity against *Alternaria alternata* strain (MIC = 5000 µg/ml). Thyme was rich in carvacrol (78.54%) and had a MIC of 6000 µg/ml against *A. alternata* strain. Clementine leaf essential oil was characterized by the predominance of monoterpene hydrocarbons (88.65%), and it possessed a weak antifungal activity against *A. alternata* (MIC = 8000 µg/ml). Cypress leaf essential oil was characterized by the predominance of oxygenated sesquiterpenes (60.67%), having an antifungal activity of 8000 µg/ml.

1. Introduction

Fungal infections are not just a human health problem but also greatly impact the field of agriculture (Mohammadi and Bahramikia, 2019). The emergence of resistant fungus strains may limit the use of synthetic fungicides, and some fungicides possess considerable toxicity. Hence, there is a growing public concern over synthetic molecules' increased health and environmental hazards. For this reason, alternative, safe, and natural methods that develop new antifungal agents are actively studied (Lopez-Reyes et al., 2013). Recently, there has been a great interest in using essential oils as possible natural substitutes for conventional synthetic fungicides (Elshafie and Camele, 2015). Essential oils can represent one of the most promising natural products for fungal inhibition (D'agostino et al., 2019). The essential oil preparations that possess antimicrobial activities have been the subject of many investigations resulting in screening a wide variety of plant species and have revealed structurally unique biologically active compounds (Matasyohet al., 2007). Again, the essential oils of some plants have recently been proven to be a successful eco-friendly bio-control agent (Raveau et al., 2020). Many authors have reported antimicrobial, antifungal, antioxidant, and radical-scavenging properties of essential oils (Chouhan et al., 2017) and, in some cases, a direct food-related application (Bhavaniramya et al., 2019). In recent years, plant essential oils have been widely studied as an emerging, environmentally-friendly, antibacterial substance. The main essential oils studied for their antifungal activity are thyme (*Thymus vulgaris* L.) essential oil, rich in thymol and carvacrol. It is already known to be effective against fungi infecting humans. Its antifungal activity is due to its high concentration of thymol and carvacrol (Yeddes et al., 2018). Cypress (*Cupressus sempervirens* L.) is known for its leaf essential oil, which is used to protect stored grains from

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insect infestation (Elansary et al., 2012), and for its antimicrobial properties (Yan et al., 2009; González and Marioli, 2010). Rosemary (*Rosmarinus officinalis* L.) essential oil is used for its antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer therapeutic properties. This essential oil also showed antimicrobial activity against various microorganisms, including pathogenic fungi (Moore et al., 2016). Already known for its antibacterial action (Golab and Skwarlo-Sonta, 2007), tea tree essential oil (*Melaleuca alternifolia* Sm.) also has an antifungal effect that was successfully evaluated in dermatomycological infections (Carson et al., 2006). Citrus leaf essential oils showed an important antimicrobial activity against some tested organisms. All citrus leaf essential oils were more effective against the fungus *Kluyveromyces fragilis* than the other microorganisms (Kasali et al., 2011).

Although a large variety of plants have been studied for their antimicrobial activities worldwide, the antifungal activities of leaf essential oils from these species are still scarce against *Alternaria alternata*. *A. alternata* is one of the most common saprophytes worldwide (Mohammadi and Bahramikia, 2019). *A. alternata* causes various diseases with an economic impact on a large range of crops, such as potato, pomegranate, almond, kiwi, cactus, tomato, ginseng, citrus, banana, and pepper water hyacinth (Dube, 2014). Therefore, this study aimed to screen the antifungal activities of the leaf essential oils from clementine, cypress, rosemary, tea, and thyme against *A. alternata* strain.

2. Materials and methods

2.1. Plant material

The samples were taken from clementine (*Citrus clementina* Hort.) at Beni Khaled (Cap Bon region, North-East Tunisia), cypress (*C. semperviens* L.) at Bir Hlima (Zaghouan region-North-West Tunisia), rosemary (*R. officinalis* L.) at Djebel Zaghouan (Zaghouan region, North-West Tunisia), tea (*M. linariifolia* Sm.) in Tunis botanical park (Tunis region-North-East Tunisia), and thyme (*T. vulgaris* L.) at Djebel Zaghouan (Zaghouan region-North-West Tunisia) during spring-summer 2019.

2.2. Essential oil extraction and analysis

The fresh leaves of each species (100 g) were submitted to hydrodistillation for 180 min using a Clevenger-type apparatus. The essential oils obtained were dried over anhydrous sodium sulfate and stored at -20 °C in darkness until analyzed (Yeddes et al., 2022a).

Analysis of volatile compounds by gas chromatography (GC) was carried out on a Hewlett-Packard 6890 GC (Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and an electronic pressure control injector. A polar polyethylene glycol HP Innowax, a 5% diphenyl, and 95% dimethylpolysiloxane apolar HP-5 capillary columns were used.

Volatile compounds analysis by gas chromatography/mass spectrometry (GC/MS) was performed on a gas chromatograph HP 5890 (II) interfaced with an HP 5972 mass spectrometer (Palo Alto, CA, USA) with electron impact ionization (70 eV).

Identification of essential oil volatile compounds was based on calculating their retention indices (RI) relative to (C_8-C_{22}) *n*-alkanes with those of authentic compounds available in our laboratory. Further identification was made by matching their recorded mass spectra with those stored in the GC-MS data systems' Wiley/NBS mass spectral library and other published mass spectra.

2.3. Fungal strain

A. alternata strain was an isolate of 7025 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5, 99.4% (*C. reticulata* - Fortune variety, Beni Khaled) GenBank accession number for nucleotide sequence: (T1): OK448177 (Grati Affes et al., 2022).

2.4. Minimum inhibitory concentration of essential oils

A portion of *A. alternata* culture stored at -20 °C in a 20% glycerol solution was taken and cultured on potato dextrose agar (PDA) in the dark at 27 °C. The method of Zerigui and Mouzaoui (2018) was applied to stimulate sporulation. After 10 days of incubation, the petri dishes were put at 4 °C for one hour. Then, they were exposed to direct light for 3 hours and returned to room temperature for 24 hours in the dark. In the end, the spore suspension was recovered after scraping the culture of *A. alternata*.

The spore suspension of *A. alternata* in a .01% Tween 80 medium was prepared from an 8-day-old single-spherical culture of several eppendorf tubes with different doses of essential oils from 0 to 12 mg/ml (diluted with a 0.5% Tween 20 solution) in potato dextrose broth (PDB) medium. Then, 20 µl of spore suspension (10^{-5}) was added. Finally, the tubes were incubated with shaking in the dark at 25 °C. The MIC was defined as the lowest concentration of the essential oil required to completely prevent visible fungal growth (Grati Affes et al., 2022).

2.5. Statistical analysis

All experiments were conducted in triplicates, and the results were expressed as mean values of standard deviation (SD). Data were subjected to statistical analysis using SAS (V.9.1). One-way analysis of variance (ANOVA) followed by Student Newman Keulstests at the significance level of 5% was used to compare means. The principal component analysis (realized by XLSTAT-2017) was used to comprehend the similarity among citrus essential oils and their antifungal activities.

3. Results and discussion

3.1. Essential oil analysis

The leaf essential oil yields of clementine, cypress, rosemary, tea, and thyme leaves were 0.22, 0.87, 1.46, 1.20, and 0.72%, respectively, based on the dry weight of the plant material. GC-MS analysis of citrus, cypress, rosemary, tea, and thyme essential oils are given in Figure 1.

As shown in Table 1, nineteen volatile compounds were identified in clementine leaf representing 94.80% of essential oil, twenty-four compounds in cypress representing 98.96% of essential oil, twenty-two compounds in rosemary representing 99.97% of essential oil, eighteen compounds in tea representing 99.92% of essential oil, and sixteen compounds in thyme representing 99.95% of essential oil. Clementine leaf essential oil was characterized by the predominance of sabinene (29.90%), limonene (28.73%), linalool (14.62%), and Δ-3-carene (7.70%). Cypress leaf essential oil was rich in germacrene-D (34.26%) and α-pinene (22.71%), while tea essential oil mainly contained 1,8-cineole (49.98%) and α-terpineol (35.92%). 1,8-

Cineole (57.55%) was the main component in rosemary essential oil and thymol (78.54%) in thyme essential oil.

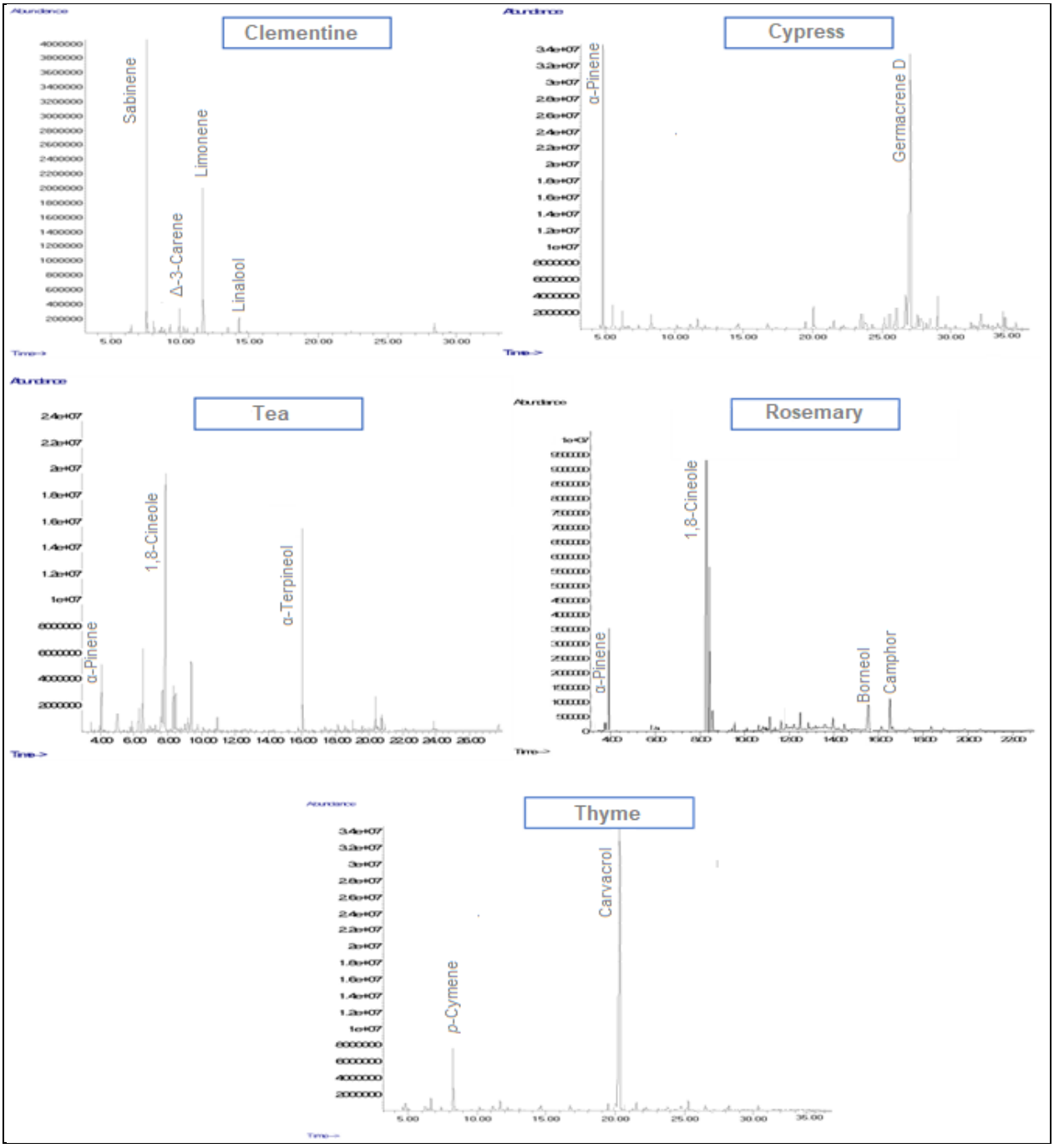

Figure 1. Gas chromatography chromatograms of the leaf essential oils from clementine, cypress, rosemary, tea, and thyme and their main volatile compounds

Fewer studies have determined the essential oil composition of clementine leaf essential oil, which was dominated by sabinene up to 36.80% (Dugo et al., 2011; Germanà et al., 2013; Thi Nguyen et al., 2016). The major component of cypress leaf essential oil was usually considered α-pinene (37.14-73.75%), as reported by Khadidja et al. (2010), Boukhris et al. (2012), Amri et al. (2013) and Hosni et al. (2019). As mentioned in the literature, rosemary leaf essential oil revealed the existence of several chemotypes as 1,8-cineole (Napoli et al., 2010; Guetat et al., 2014; Yeddes et al., 2018; Yeddes et al., 2022a), camphor (Celiktas et al., 2007; Zaouali and Boussaid, 2008; Lakušić et al., 2012), verbenone (Mata et al., 2007; Papageorgiou et al., 2008; Varela et al., 2007), α-pinene (Angioni et al., 2004; Napoli et al., 2010), linalool (Varela et al., 2007), and *p*-cymene (Özcan and Chalchat, 2008) chemotypes. Tea leaf essential oil was characterized by its richness in 1,8-cineole (61.10-77.40%) and α-terpineol (7.72-12.30%) in India (Padalia et al., 2015; Joshi et al., 2022). However, Australian tea leaf essential oil showed α-terpineol (30.18%) and γ-terpinene (15.20%) chemotype (Park et al., 2011). The essential oil of tea leaf from Brazil was shown to be characterized by methyl eugenol (86.8%) and *(E)*-methyl isoeugenol (1.4%) (Silva et al., 2010). Thyme leaf essential oil was rich in carvacrol ranging from 20 to 71% (Porte and Godoy, 2008; Aslam et

al., 2022; Yeddes et al., 2022b). Numerous studies also reported the essential oil composition of thyme with thymol as the main constituent ranging from 22 to 71% (Soković et al., 2009; Shabnum and Wagay, 2011; Kowalczyk et al., 2020). These variations in the essential oil compositions between the same species could be attributed to many factors such as genetic dissimilarity within accessions, climate variability, plant origin, sample drying, storage, and extraction processes (Sadeh et al., 2019).

Table 1. Essential oil compositions of clementine, cypress, rosemary, tea, and thyme leaves*

Volatile compounds	RI[a]	RI[b]	Clementine	Cypress	Rosemary	Tea	Thyme
α-Thujene	923	836	0.17 ± 0.08[a]	0.08 ± 0.04[c]	0.11 ± 0.12[b]	0.08 ± 0.04[c]	-
α-Pinene	934	982	0.65 ± 0.22[e]	22.71 ± 0.04[a]	8.83 ± 0.14[b]	2.96 ± 0.79[c]	1.07 ± 0.22[d]
β-Pinene	937	1113	-	0.08 ± 0.04[c]	2.82 ± 0.36[a]	0.30 ± 0.14[b]	-
Camphene	952	1077	-	-	2.36 ± 0.19[a]	0.13 ± 0.06[c]	0.31 ± 0.06[b]
Sabinene	983	1111	29.90 ± 0.22[a]	-	0.04 ± 0.02[c]	-	0.16 ± 0.03[b]
β-Myrcene	991	1168	1.58 ± 0.36[b]	0.18 ± 0.08[e]	0.89 ± 0.36[c]	1.65 ± 0.56[a]	0.58 ± 0.12[d]
α-Phellandrene	1005	1025	0.23 ± 0.10[a]	-	0.19 ± 0.20[b]	-	-
Δ-3-Carene	1011	1159	7.70 ± 0.23[a]	2.05 ± 0.36[b]	0.08 ± 0.04[c]	-	-
α-Terpinyl acetate	1014	1340	-	2.61 ± 0.48[a]	-	-	-
α-Terpinene	1018	1255	-	-	0.62 ± 0.16[b]	-	0.91 ± 0.19[a]
α-Cubebene	1352	1460	-	0.76 ± 0.23[a]	-	-	-
p-Cymene	1026	1277	-	-	1.01 ± 0.36[b]	0.07 ± 0.04[c]	7.13 ± 1.49[a]
Limonene	1030	1031	28.73 ± 0.67[a]	-	-	0.04 ± 0.02[b]	-
1,8-cineole	1033	1029	-	-	57.55 ± 1.54[a]	49.98 ± 3.27[b]	3.50 ± 0.73[c]
(*E*)-β-Ocimene	1052	1022	4.20 ± 0.43[a]	-	-	-	-
γ-Terpinene	1059	1262	0.89 ± 0.27[d]	-	1.00 ± 0.09[c]	1.56 ± 0.54[b]	3.44 ± 0.72[a]
Terpinolene	1083	1086	1.52 ± 0.36[a]	0.41 ± 0.15[b]	0.34 ± 0.05[c]	-	-
Fenchol	1121	1126	-	-	-	0.41 ± 0.19[a]	-
Linalool	1098	1551	14.62 ± 0.53[a]	-	0.57 ± 0.09[b]	-	0.39 ± 0.08[c]
Citronellal	1151	1542	3.72 ± 0.42[a]	-	-	-	-
Borneol	1165	1642	-	-	5.54 ± 1.12[a]	-	0.85 ± 0.18[b]
Terpinen-4-ol	1178	1593	2.68 ± 0.43[a]	-	1.02 ± 0.31[b]	0.50 ± 0.22[d]	0.64 ± 0.13[c]
α-Terpineol	1185	1772	0.41 ± 0.16[c]	-	3.62 ± 0.68[b]	35.93 ± 0.92[a]	0.14 ± 0.03[d]
Citronellol	1224	1624	0.69 ± 0.23[a]	-	-	-	-
Geraniol	1271	1856	0.95 ± 0.25[a]	-	-	-	-
Eugenol	1344	1752	-	-	-	0.20 ± 0.09[a]	-
Camphor	1192	1498	-	-	8.82 ± 1.09[a]	-	-
Bornyl acetate	1285	1291	-	-	0.82 ± 0.08[a]	-	-
Thymol	1296	2198	-	-	-	-	0.18 ± 0.04[a]
Carvacrol	1306	2239	-	-	-	-	78.54 ± 4.50[a]
Isoledene	1376	1687	-	0.56 ± 0.19[a]	-	-	-
α-Copaene	1395	1391	-	0.28 ± 0.12[a]	-	0.09 ± 0.04[b]	-
Methyl eugenol	1401	1405	-	-	-	0.49 ± 0.22[a]	-
α-Cedrene	1410	1577	-	1.30 ± 0.31[a]	-	-	-
(*E*)-Caryophyllene	1446	1608	0.69 ± 0.23[d]	3.94 ± 0.37[a]	1.99 ± 009[b]	0.06 ± 0.03[e]	1.58 ± 0.33[c]
cis-muurola-3.5-diene	1465	1699	-	0.84 ± 0.25[a]	-	-	-
Germacrene D	1480	1685	-	34.26 ± 3.89[a]	-	-	-
α-Humulene	1485	1691	0.08 ± 0.04[b]	-	0.29 ± 0.02[a]	0.04 ± 0.02[c]	-
Longipinene	1489	1692	-	0.81 ± 0.24[a]	-	-	-
Bicyclogermacrène	1490	1757	0.18 ± 0.17	-	-	-	-
epi-Bicyclosesquiphellandrene	1492	1702	-	2.67 ± 0.38[a]	-	-	-
γ-Muurolene	1502	1704	-	2.80 ± 0.38[a]	-	-	-
β-Cadinene	1525	1776	-	6.66 ± 0.21[a]	-	-	-
γ-Cadinene	1532	1530	-	1.60 ± 0.34[a]	-	-	-
α-Caryophyllene	1564	1677	-	2.98 ± 0.38[a]	-	-	-
Caryophyllene oxide	1578	1582	-	0.18 ± 0.06[c]	0.20 ± 0.10[c]	0.49 ± 0.22[b]	0.57 ± 0.12[a]
α-Cedrol	1608	1616	-	9.17 ± 0.03[a]	-	-	-
α-Cadinol	1654	1546	-	0.45 ± 0.16[a]	-	-	-
Sclareol	1659	1555	-	0.39 ± 0.15[a]	-	-	-
Monoterpene hydrocarbons			88.65 ± 1.12[a]	25.51 ± 2.38[c]	26.99 ± 0.73[b]	6.79 ± 0.72[e]	13.60 ± 0.25[d]
Oxygenated monoterpenes			4.27 ± 0.75[d]	2.61 ± 0.22[e]	60.14 ± 0.66[c]	91.70 ± 2.15[a]	84.20 ± 1.75[b]
Sesquiterpene hydrocarbons			1.88 ± 0.09[b]	10.17 ± 0.10[a]	0.56 ± 0.04[c]	0.49 ± 0.10[d]	0.57 ± 0.05[c]
Oxygenated sesquiterpenes			-	60.67 ± 0.90[a]	12.28 ± 0.06[b]	0.94 ± 0.16[d]	1.58 ± 0.09[c]
Total			94.80 ± 1.32[c]	98.96 ± 2.30[b]	99.97 ± 1.12[a]	99.92 ± 0.99[a]	99.95 ± 1.32[a]

*Compounds in order of elution on HP-5 MS.
[a]RI: Retention index calculated on HP-5 MS column.
[b]RI: Retention index calculated on HP Innowax column.
Means of three replicates (Values with different superscripts are significantly different at $p < 0.05$).

3.2. In vitro antifungal activity

The antifungal activities of leaf essential oils from clementine, cypress, rosemary, tea, and thyme species were tested against *A. alternata* strain. The minimal concentration of these essential oils ranged between 5000 and 8000 µg/ml, completely inhibited the growth of *A. alternata*. The results presented in Table 2 showed similar activity between clementine and cypress essential oils with MIC = 8000 µg/ml. A similar result was obtained between rosemary and tea essential oils with MIC = 5000 µg/ml. The MIC of thyme essential oil was 6000 µg/ml. So, rosemary and tea essential oils had the best antifungal activity against *A. alternata* strain. Contrarily to our results, Shaban (2014) reported that the highest degree of antifungal activity against *A. alternata* was caused by thyme essential oil and followed by rosemary essential oil. Amri et al. (2013) found that cypress leaf essential oil had potent antifungal activity against *Alternaria* spp. (75.21%). Alves et al. (2019) detected that tea (*M. alternifolia*) essential oil had a strong antifungal activity

(MIC = 14.49 µg/ml) against *A. alternata*. Hamdani and Allem (2015) determined the antifungal activity of the leaf essential oil from citrus (*C. limon*, *C. sinensis*, and *C. reticulata*) and found that the concentration of 1000 µg/ml was sufficient to inhibit the development of *A. alternata*. This difference in results could be due to the existent differences in components and the concentrations of the active compounds in the essential oils of each species.

Table 2. Antifungal activity of leaf essential oils from clementine, cypress, rosemary, tea and thyme

Species	Minimum inhibitory concentration (MIC, µg/ml)				
	Clementine	Cypress	Rosemary	Tea	Thyme
A. alternata	8000 ± 0.00[a]	8000 ± 0.00[a]	5000 ± 0.00[c]	5000 ± 0.00[c]	6000 ± 0.00[b]

Values with different superscripts are significantly different at $p < 0.05$.

Table 3. Correlation between the main essential oil compounds, essential oil yield, and antifungal activity

	α-Pinene	Sabinene	Δ-3-Carene	*p*-Cymene	Limonene	1,8-Cineole	Linalool	α-Terpineol	Carvacrol	EO yield	Strain
α-Pinene	11										
Sabinene	0.095	1									
Δ-3-Carene	0.809	0.453	1								
p-Cymene	-0.073	-0.309	-0.405	1							
Limonene	-0.796	0.000	-0.348	-0.296	1						
1,8-Cineole	0.747	-0.309	0.704	-0.250	-0.297	1					
Linalool	-0.073	-0.237	-0.405	-0.250	-0.284	-0.249	1				
α-Terpineol	0.723	-0.010	0.850	-0.528	-0.183	0.934	-0.255	1			
Carvacrol	-0.801	-0.245	-0.405	-0.250	0.990	-0.250	-0.250	-0.179	1		
EO yield	-0.256	0.687	-0.216	-0.028	-0.110	0.794	0.361	-0.611	-0.205	1	
Strain	0.560	0.587	0.145	0.590	-0.232	-0.589	-0.516	0.306	-0.547	-0.709	1

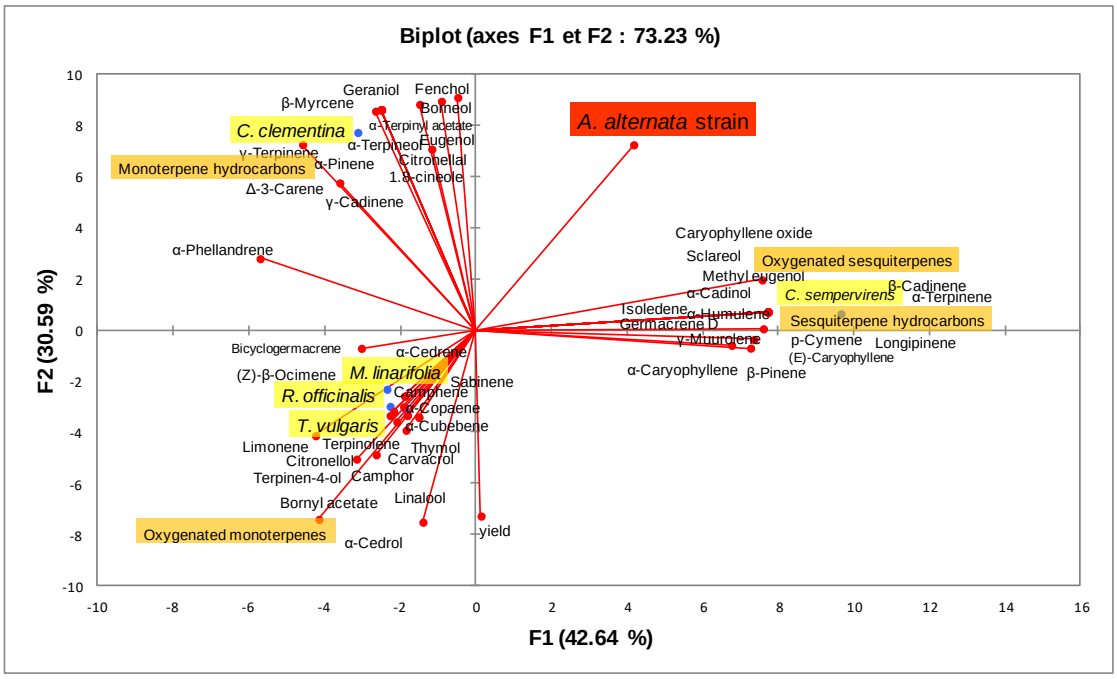

Figure 2. Biplot obtained from principal component analysis of variables comprising essential oil components, chemical classes, and antifungal activities

3.3. Relation between essential oil compounds and antifungal activity

The analysis of Pearson's correlation coefficients between the main essential oil compounds and antifungal activity (Table 3) showed a positive correlation between *A. alternata* strain and the volatile compounds α-pinene (r = 0.560), sabinene (r = 0.587), and *p*-cymene (r = 0.590). This could be explicated by the fact that the high proportions of α-pinene in cypress and sabinene in clementine contributed to the high MIC values of these two species (MIC = 8000 µg/ml). However, there was a negative correlation between *A. alternata* strain and the volatile compounds 1,8-cineole (r = -0.589), linalool (r = -0.516) and carvacrol (r = -0.547). So, the high proportions of carvacrol in thyme and 1,8-cineole in rosemary and tea contributed to the low MIC values of these species (MIC = 6000 µg/ml for thyme and 5000 µg/ml for rosemary and tea). On the other hand, there was a strong negative correlation between the essential oil yield and *A. alternata* strain (r = -0.709), explicating that the antifungal activity depended on the yield of these essential oils. On the other hand, there was a strong correlation between these volatile compounds as between α-pinene/Δ-3-carene (0.809), α-pinene/limonene (r = -0.796), α-pinene/1,8-cineole (r = 0.747), α-pinene/α-terpineol (r = 0.723), α-pinene/carvacrol (r = -0.801), Δ-3-carene/1,8-cineole (r = 0.704), Δ-3-carene/α-terpineol (r = 0.850), *p*-cymene/α-terpineol (r = -0.528), limonene/carvacrol (r = -0.990), and 1,8-cineole/α-terpineol (r = 0.934). So, there is a relationship in the biosynthesis of these compounds, known as monoterpenes, via

a universal precursor of monoterpenes is geranyl pyrophosphate, combining two C5 units, which is then further processed by monoterpene synthases/cyclases to produce a vast array of chemical structures (Reiling et al., 2004).

3.4. Principal component analysis

The principal component analysis (PCA) was applied to assess the chemical composition and the antifungal activity of leaf essential oils from clementine, cypress, tea, rosemary, and thyme species (Figure 2). So, PCA greatly helps interpret results from the experiments, the two-dimensional axial systems generated from PCA of these essential oils showed that there were three main groups, as indicated in Figure 2. In fact, rosemary, tea, and thyme were closer due to the similarities of the highest levels of oxygenated monoterpenes (60.14-91.70%). Rosemary and tea were rich in 1,8-cineole (49.98% and 57.55%, respectively), and they have potent antifungal activity against *A. alternata* strain (MIC = 5000 µg/ml). Thyme was rich in carvacrol (78.54%) and had a MIC of 6000 µg/ml against *A. alternata* strain. Clementine leaf essential oil was characterized by the predominance of monoterpene hydrocarbons (88.65%), and it possessed a weak antifungal activity against *A. alternata* (MIC = 8000 µg/ml), constituting the second cluster. Finally, cypress leaf essential oil formed the third cluster characterized by the predominance of oxygenated sesquiterpenes (60.67%), having an antifungal activity of 8000 µg/ml. Accordingly to Bassolé et al. (2010), phenolic monoterpenes (thymol and carvacrol) and phenylpropanoids (eugenol) in combination with other components were found to increase the bioactivities of essential oils. Most studies have focused on the interaction of phenolic monoterpenes and phenylpropanoids with other groups of components, particularly with other phenols, phenylpropanoids, and monoterpenes alcohols, while monoterpenes and sesquiterpenes hydrocarbons were used to a lesser extent. Socović et al. (2009) found that thyme essential oil and its phenolic components (carvacrol and thymol) had very high antifungal activities, even higher than the commercial fungicide bifonazole. These authors deduced a relationship between the high activity of thyme essential oil and the presence of phenol components, such as thymol and carvacrol. It seems possible that phenol components may interfere with cell wall enzymes like chitin synthase/chitinase as well as with the α- and β-glucanases of the fungus (Adams et al., 1996).

4. Conclusions

In summary, the leaf essential oils of rosemary and tea had the highest antifungal activity, followed by thyme, cypress, and clementine essential oils. Rosemary and tea leaf essential oils were rich in 1,8-cineole. Carvacrol was the main component of thyme essential oil, while cypress leaf essential oil mainly contained germacrene-D and α-pinene. Clementine leaf essential oil was characterized by the predominance of sabinene, limonene, linalool, and Δ-3-carene. It was difficult to attribute the antifungal activity of essential oils to a single component. Possible combinations between these essential oils could be conducted in further investigations to determine these bioactive compounds' synergistic and antagonistic effects. These investigations could lead to the development of a new treatment based on combining these essential oils as natural bioactive substances against *A. alternata*.

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Conflict of interest

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CRediT authorship contribution statement

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Supplementary File

None.

References

Adams, S., Kunz, B., Weidenbörner, M., 1996. Mycelial deformations of *Cladosporium herbarum* due to the application of eugenol or carvacrol. *Journal of Essential Oil Research*, 8(5), 535-540.

Alves, F.M.F., França, K.R.S., de Araújo, I.G., da Nóbrega, L.P., Xavier A.L.S., Lima T.S., Rodrigues, A.P.M.S., Júnior, A.F.M., Cardoso, T.A.L., 2019. Control of *Alternaria alternata* using Melaleuca essential oil (*Melaleuca alternifolia*). *Journal of Experimental Agriculture International*, 40(3), 1-10.

Amri, I., Hamrouni, L., Hanana, M., Gargouri, S., Jamoussi, B., 2013. Chemical composition, bio-herbicidal and antifungal activities of essential oils isolated from Tunisian common cypress (*Cupressus sempervirens* L.). *Journal of Medicinal Plants Research*, 7(16), 1070-1080.

Angioni, A., Barra, A., Cereti, E., Barile, D., Coïsson, J.D., Arlorio, M., Cabras, P., 2004. Chemical composition, plant genetic differences, antimicrobial and antifungal activity investigation of the essential oil of *Rosmarinus officinalis* L. *Journal of Agricultural and Food Chemistry*, 52(11), 3530-3535.

Aslam, M.F., Irshad, G., Naz, F., Khan, M.A., 2022. Evaluation of the antifungal activity of essential oils against *Alternaria alternata* causing fruit rot of *Eriobotrya japonica*. *Turkish Journal of Biochemistry*, https://doi.org/10.1515/tjb-2021-0225.

Bassolé, I.H.N., Lamien-Meda, A., Bayala, B., Tirogo, S., Franz, C., Novak, J., Dicko, M.H., 2010. Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha x piperita* L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. *Molecules*, 15(11), 7825-7839.

Bhavaniramya, S., Vishnupriya, S., Al-Aboody, M.S., Vijayakumar, R., Baskaran, D., 2019. Role of essential oils in food safety: Antimicrobial and antioxidant applications. *Grain & Oil Science and Technology*, 2(2), 49-55.

Boukhris, M., Regane, G., Yangui, T., Sayadi, S., Bouaziz, M., 2012. Chemical composition and biological potential of essential oil from Tunisian *Cupressus sempervirens* L. *Journal of Arid Land Studies*, 22(1), 329-332.

Carson, C.F., Hammer, K.A., Riley, T.V., 2006. *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clinical Microbiology Reviews*, 19(1), 50-62.

Celiktas, O.Y., Kocabas, E.H., Bedir, E., Sukan, F.V., Ozek, T., Baser, K.H.C., 2007. Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations. *Food Chemistry*, 100(2), 553-559.

Chouhan, S., Sharma, K., Guleria, S., 2017. Antimicrobial activity of some essential oils—present status and future perspectives. *Medicines*, 4(3), 58.

D'agostino, M., Tesse, N., Frippiat, J.P., Machouart, M., Debourgogne, A., 2019. Essential oils and their natural active compounds presenting antifungal properties. *Molecules*, 24(20), 3713.

Dube, J.P., 2014. Characterization of *Alternaria alternata* isolates causing brown spot of potatoes in South Africa (Master Thesis in Science (Agriculture) Plant Pathology). University of Pretoria. (accessed 2014 February).

Dugo, G., Cotroneo, A., Bonaccorsi, I., Trozzi, A., 2011. Composition of the volatile fraction of citrus peel oils, Chapter 1, 1–161; Dugo, G., Cotroneo, A., Bonaccorsi, I. Composition of petitgrain oils, Chapter 5, 253-331. In citrus oils: Composition, advanced analytical techniques, contaminants, and biological activity. Edits. Dugo, Mondello, L., CRC Press - Taylor & Francis Group, Bacon Raton.

Elansary, H.O., Salem, M.Z., Ashmawy, N.A., Yacout, M.M., 2012. Chemical composition, antibacterial and antioxidant activities of leaves essential oils from *Syzygium cumini* L., *Cupressus sempervirens* L. and *Lantana camara* L. from Egypt. *Journal of Agricultural Science*, 4(10), 144-152.

Elshafie, H.S., Camele, I., 2017. An overview of the biological effects of some mediterranean essential oils on human health. *BioMed Research International*, 2017, 9268468.

Germanà, M.A., Palazzolo, E., Chiancone, B., Saiano, F., 2013. Characterization of leaf essential oil composition of homozygous and heterozygous *Citrus clementina* Hort. Extan. and its Ancestors. *Journal of Essential Oil Bearing Plants*, 16(1), 92-101.

Golab, M., Skwarlo-Sonta, K., 2007. Mechanisms involved in the anti-inflammatory action of inhaled tea tree oil in mice. *Experimental Biology and Medicine*, 232(3), 420-426.

González, M.J., Marioli, J.M., 2010. Antibacterial activity of water extracts and essential oils of various aromatic plants against *Paenibacillus* larvae, the causative agent of American Foulbrood. *Journal of Invertebrate Pathology*, 104(3), 209-213.

Grati Affes, T., Chenenaoui, S., Zemni, H., Hammami, M., Bachkouel, S., Aidi Wannes, W., Lasram, S., 2022. Biological control of *Citrus* brown spot pathogen,"*Alternaria alternata*" by different essential oils. *International Journal of Environmental Health Research*, 1-14.

Guetat, A., Al-Ghamdi, F.A., Osman, A.K., 2014. 1, 8-Cineole, α-Pinene and Verbenone chemotype of essential oil of species *Rosmarinus officinalis* L. from Saudi Arabia. *International Journal of Herbal Medicine*, 2, 137-141.

Hamdani, F.Z., Allem, R., 2015. Antifungal activity of the leaf essential oil of citrus against *Alternaria alternata in vivo*. Internat*ional Journal of Applied and Natural Sciences*, 4(4), 15-24.

Hosni, K., Hassen, I., M'Rabet, Y., Casabianca, H., 2019. Biochemical response of *Cupressus sempervirens* to cement dust: Yields and chemical composition of its essential oil. *Arabian Journal of Chemistry*, 12(7), 1308-1314.

Joshi, A., Prakash, O., Pant, A.K., Kumar, R., Szczepaniak, L., Kucharska-Ambrożej, K., 2022. Methyl eugenol, 1, 8-cineole and nerolidol rich essential oils with their biological activities from three melaleuca species growing in Tarai region of North India. Brazilian *Archives of Biology and Technology*, 64, e21210186.

Kasali, A.A., Lawal, O.A., Eshilokun, A.O., Olaniyan, A.A., Opoku, A.R., Setzer, W.N., 2011. *Citrus* essential oil of Nigeria part V: Volatile constituents of sweet orange leaf oil (*Citrus sinensis*). *Natural Product Communications*, 6(6), 875-878.

Khadidja, M., Nassima, B., Chahrazed, B., Xavier, F., 2010. Chemical composition and antimicrobial activity of essential oils isolated from Algerian *Juniperus phoenicea* L. and *Cupressus sempervirens* L. *Journal of Medicinal Plants Research*, 4(10), 959-964.

Kowalczyk, A., Przychodna, M., Sopata, S., Bodalska, A., Fecka, I., 2020. Thymol and thyme essential oil—new insights into selected therapeutic applications. *Molecules*, 25(18), 4125.

Lakušić, D.V., Ristić, M.S., Slavkovska, V.N., Šinžar-Sekulić, J.B., Lakušić, B.S., 2012. Environment-Related Variations of the Composition of the Essential Oils of Rosemary (*Rosmarinus officinalis* L.) in the Balkan Penninsula. *Chemistry & Biodiversity*, 9(7), 1286-1302.

Lopez-Reyes, J.G., Spadaro, D., Preile, A., Garibaldi, A., Gullino, M.L., 2013. Efficacy of plant essential oils on postharvest control of rots caused by fungi on different stone fruits *in vivo*. *Journal of Food Protection*, 76, 631-639.

Mata, A.T., Proença, C., Ferreira, A.R., Serralheiro, M.L M., Nogueira, J.M.F., Araújo, M.E.M., 2007. Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices. *Food Chemistry*, 103(3), 778-786.

Matasyoh, J.C., Kiplimo, J.J., Karubiu, N.M., Hailstorks, T.P., 2007. Chemical composition and antimicrobial activity of the essential oil of *Satureja biflora* (Lamiaceae). *Bulletin of the Chemical Society of Ethiopia*, 21(2), 249-254.

Mohammadi, A., Bahramikia, S., 2019. Molecular identification and genetic variation of *Alternaria* species isolated from tomatoes using ITS1 sequencing and inter simple sequence repeat methods. *Current Medical Mycology*, 5(2), 1-8.

Moore, J., Yousef, M., Tsiani, E., 2016. Anticancer effects of rosemary (*Rosmarinus officinalis* L.) extract and rosemary extract polyphenols. *Nutrients*, 8(11), 731.

Napoli, E.M., Curcuruto, G., Ruberto, G., 2010. Screening of the essential oil composition of wild Sicilian rosemary. *Biochemical Systematics and Ecology*, 38(4), 659-670.

Özcan, M.M., Chalchat, J.C., 2008. Chemical composition and antifungal activity of rosemary (*Rosmarinus officinalis* L.) oil from Turkey. *International Journal of Food Sciences and Nutrition*, 59(7-8), 691-698.

Padalia, R.C., Verma, R.S., Chauhan, A., Chanotiya, C.S., 2015. The essential oil composition of *Melaleuca leucadendra* L. grown in India: A novel source of *(E)*-nerolidol. *Industrial Crops and Products*, 69, 224-227.

Papageorgiou, V., Gardeli, C., Mallouchos, A., Papaioannou, M., Komaitis, M., 2008. Variation of the chemical profile and antioxidant behavior of *Rosmarinus officinalis* L. and *Salvia fruticosa* Miller grown in Greece. *Journal of Agricultural and Food Chemistry*, 56(16), 7254-7264.

Park, H.M., Kim, J., Chang, K.S., Kim, B.S., Yang, Y.J., Kim, G.H., Park, I.K., 2011. Larvicidal activity of Myrtaceae essential oils and their components against *Aedes aegypti*, acute toxicity on *Daphnia magna*, and aqueous residue. *Journal of Medical Entomology*, 48(2), 405-410.

Porte, A., Godoy, R.L., 2008. Chemical composition of *Thymus vulgaris* L.(Thyme) essential oil from the Rio de Janeiro state, Brazil. *Journal of the Serbian Chemical Society*, 73(3), 307-310.

Raveau, R., Fontaine, J., Lounès-Hadj Sahraoui, A., 2020. Essential oils as potential alternative biocontrol products against plant pathogens and weeds: A review. *Foods*, 9(3), 365.

Reiling, K.K., Yoshikuni, Y., Martin, V.J., Newman, J., Bohlmann, J., Keasling, J.D., 2004. Mono and diterpene production in *Escherichia coli*. *Biotechnology and Bioengineering*, 87(2), 200-212.

Sadeh, D., Nitzan, N., Chaimovitsh, D., Shachter, A., Ghanim, M., Dudai, N., 2019. Interactive effects of genotype, seasonality and extraction method on chemical compositions and yield of essential oil from rosemary (*Rosmarinus officinalis* L.). *Industrial Crops and Products*, 138, 111419.

Shaban, W., 2014. Evaluation of some fungicide alternatives against *Alternaria* fruit rot on tomato. *Egyptian Journal of Phytopathology*, 42(2), 91-104.

Shabnum, S., Wagay, M.G., 2011. Essential oil composition of *Thymus vulgaris* L. and their uses. *Journal of Research and Development*, 11, 83-94.

Silva, M.R., Ximenes, R.M., da Costa, J.G.M., Leal, L.K.A.M., de Lopes, A.A., de Barros Viana, G.S., 2010. Comparative anticonvulsant activities of the essential oils (EOs) from *Cymbopogon winterianus* Jowitt and *Cymbopogon citratus* (DC) Stapf. in mice. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 381(5), 415-426.

Soković, M.D., Vukojević, J., Marin, P.D., Brkić, D.D., Vajs, V., Van Griensven, L.J., 2009. Chemical composition of essential oils of *Thymus* and *Mentha* species and their antifungal activities. *Molecules*, 14(1), 238-249.

Thi Nguyen, T.T., Thi Tran, T.T., Hua, T.M., Diep, T.T., Chau, D.K.N., Duus, F., Le, T.N., 2016. Investigation of peel and leaf essential oils of *Citrus clementina* Hort. ex Tan. growing in the south of Vietnam. *Journal of Essential Oil Research*, 28(2), 96-103.

Varela, F., Navarrete, P., Cristobal, R., Fanlo, M., Melero, R., Sotomayor, J.A., Cases, A., 2007. Variability in the chemical composition of wild *Rosmarinus officinalis* L. *In I International Medicinal and Aromatic Plants Conference on Culinary Herbs*, 826, 167-174.

Yan, R., Yang, Y., Zeng, Y., Zou, G., 2009. Cytotoxicity and antibacterial activity of *Lindera strychnifolia* essential oils and extracts. *Journal of Ethnopharmacology*, 121(3), 451-455.

Yeddes, W., Aidi Wannes, W., Hammami, M., Smida, M., Chebbi, A., Marzouk, B., Saidani Tounsi, M., 2018. Effect of environmental conditions on the chemical composition and antioxidant activity of essential oils from *Rosmarinus officinalis* L. growing wild in Tunisia. *Journal of Essential Oil Bearing Plants*, 21(4), 972-986.

Yeddes, W., Hammami, M., Khammassi, S., Grati Affes, T., Aidi Wannes, W., Saidani-Tounsi, M., 2022a. Antibacterial activities of rosemary (*Rosmarinus officinalis* L.) essential oil andethanol extract. *Open Access Research Journal of Multidisciplinary Studies*, 3(1), 1-8.

Yeddes, W., Mejri, I., Grati Affes, T., Khammassi, S., Hammami, M., Aidi-Wannes, W., Saidani Tounsi, M., 2022b. Combined effect of essential oils from Clove (*Syzygium aromaticum* (L.) Merr. & LM Perry), Thyme (*Thymus vulgaris* L.) and Lemon peel (*Citrus limon* (L.) Osbeck) on anti-bacterial, cytotoxic and anti-inflammatory activities. *Trends in Phytochemical Research*, 6(1), 11-18.

Zaouali, Y., Boussaid, M., 2008. Isozyme markers and volatiles in Tunisian *Rosmarinus officinalis* L.(Lamiaceae): A comparative analysis of population structure. *Biochemical Systematics and Ecology*, 36(1), 11-21.

Zerigui, F., Mouzaoui, F., 2018.Contribution à l'étude d'*Alternaria* sp., agent causal de l'alternariose de la pomme de terre : Prospection, isolement et identification du pathogène. Master research, Abdelhamid Ben Badis-Mostagane Univresity, Algeria, 49pp.

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REVIEW

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Phytochemistry, nutritional composition and pharmacological potential of *Moringa oleifera*: A comprehensive review

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ABSTRACT

Moringa oleifera is a medicinally important plant of the family Moringaceae, which is highly valued because of its enormous nutritional and therapeutic potential. Each part of *M. oleifera* is said to contain medicinal properties, which contributes to its diversity and value as a medicinal herb. Literature has revealed that the plant possesses anticancer, antimicrobial, anti-inflammatory, antidiabetic, antifertility, hepatoprotective, cardiovascular, and other important pharmacological attributes. Most of these biological activities of the plant are due to its high content of phytochemicals, including flavonoids, alkaloids, saponins, terpenes, glucosides, glucosinolates, anthocyanins, and steroids. Aside from the plant's exceptional phytochemicals and therapeutic potentials, it is also rich in essential nutrients such as vitamins, minerals, proteins, beta-carotene, fiber, and fatty acids. This systematic review aims at providing updated and categorized scientific data on phytochemicals, nutritional composition, and pharmacological potentials of *M. oleifera*. The information compiled in this present review would be crucial for developing novel therapeutic medicines and pharmaceutical formulations, which are more effective with great curative actions.

1. Introduction

Plants with medicinal properties have been utilized to cure human ailments since the genesis of humanity. The upsurge of interest in natural pharmaceuticals began around a decade ago, owing to the belief that natural medicine is safer than synthetic medicine (Kenwat et al., 2013). Due to the growing interest in the use of medical plants around the world, which is growing at a pace of 7 to 15% yearly, there has been a significant increase in medicinal plant-based industries (Kumar, 2013). Medicinal plants are used by roughly 70-80 percent of the world's people, mainly in developing nations, to combat their health regiments (Ekor, 2014), and about 25% of synthesized pharmaceuticals are made from medicinal plants (Pan et al., 2013).

Herbal medications and their constituents are essential in several therapeutic systems such as Unani, Siddha, yoga, homeopathy, naturopathy, and Ayurveda (Paikra and Gidwani, 2017). Among the diverse medicinal plants, *Moringa oleifera* is largely utilized in many traditional systems of medicine and folklore for many diseases. What is fascinating about *M. oleifera* is that a separate part of the plant is used as medicine.

M. oleifera is generally known as the "wonder plant" or the "tree of life" (Mishra et al., 2020). This name is derived from the plant's numerous uses, particularly concerning medicine and nutrition. It is already an important crop grown for commercial purposes in locations such as India, Ethiopia, the Philippines, Sudan, the West, East, South Africa, and Asia (Mbikay, 2012). The immature seed pods are consumed in some regions, but the fresh leaves are

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generally utilized as a staple food due to their high nutritional content (Thurber and Fahey, 2009). *Moringa* is frequently regarded as an important famine food due to its high resistance to arid conditions owed to its tuberous roots (Berushka and Himansu, 2012). Virtually every part of *M. oleifera*, including leaves, flowers, bark, green pods, roots, and seeds, is beneficial for medicine, functional food preparations, nutraceuticals, water filtration, and biodiesel generation (Saini, 2013).

Many reports have appeared in mainstream scientific journals over the last two decades describing the nutritive and medicinal properties of *M. oleifera*. According to Popoola and Obembe (2013), *M. oleifera* is nutritious and beneficial to people by providing daily nutritional supplements and boosting their immune systems. Mahmood et al. (2010) reported that *Moringa* leaves are high in vitamin C, A, and essential amino acids. Abd Rani et al. (2018) and Mishra et al. (2020) also added that *Moringa* leaves contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas, and their protein quality rivals that of milk and eggs. The immature pods, flowers, and leaves of the *Moringa* tree are also utilized in cooking in several regions of the world (Stevens et al., 2013). Because of the high level of monounsaturated fatty acids (in the form of oleic acid) in moringa seed oil, it is used in biodiesel production (Azam et al., 2005).

Studies on *M. oleifera* have revealed some phytochemical substances that offer health benefits beyond the plant's basic nutritional value. Phytochemicals such as alkaloids, tannins, terpenoids, steroidal aglycones, and reducing sugars are abundant in the leaves, flowers, seeds, bark, roots, and immature pods (Paikra and Gidwani, 2017). Leone et al. (2015) stated that phenolic acids, flavonoids, polyphenols, alkaloids, glucosinolates, isothiocyanates, tannins, and saponins are all present in the leaves, which is the most often used component of the plant. *M. oleifera* leaves have also been discovered to be a significant source of phenolics and glucosinolates (Amaglo et al., 2010). The presence of a high concentration of bioactive compounds in the leaves of *M. oleifera* may explain its pharmacological properties. According to Sivasankari et al. (2014), the leaves of *M. oleifera* are used to treat a variety of diseases ranging from malaria and typhoid fever to hypertension and diabetes. The *M. oleifera* foliage also guards against inflammation, oxidative stress, hepatic fibrosis, liver damage, hypercholesterolemia, bacterial activity, and cancer (Okwari et al., 2013; Halaby et al., 2013; Efiong et al., 2013). Other plant parts, such as the roots, bark, gum, fruit (pods), flowers, and seeds, have also been shown to have biological activities such as protection against gastric ulcers, antidiabetic, hypotensive, and anti-inflammatory properties (Oyedepo et al., 2013; Vergara-Jimenez et al., 2017).

Several works have been conducted to explore the potential of *M. oleifera* in traditional systems of medicine and nutrition. However, little or no updated and a well-organized comprehensive review exists on the phytochemicals, nutritive values, and pharmacological properties of *M. oleifera*. Hence, this present review is focused on compiling the phytochemical compounds, nutritional composition, and pharmacological potentials of the various parts of *M. oleifera*. Additionally, the prospects of this important medicinal plant will be evaluated. This review work would encourage new research on *M. oleifera* by serving as valuable background for future studies.

2. Methodology

An extensive search for important literature was performed through several scientific databases, including Google Scholar, Scopus, PubMed, and ScienceDirect, for journal articles published from 2000 to 2022. This was done using the keywords of *Moringa oleifera*, phytochemistry, nutrition, and pharmacognosy. Only data from books, original research papers, and review articles from peer-reviewed journals linked to the keywords mentioned above were incorporated in the present review. The various citations were combined using the EndNote X9 citation tool (Thomson Reuters, Toronto, Canada). The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) framework for collecting data for this review is displayed in Figure 1.

3. Geographical source

M. oleifera is a rapidly growing tree widely cultivated throughout India's plains and has naturalized in tropical areas. Sri Lanka, Northern Pakistan, Bangladesh, the Arabian Peninsula, Asia, West and East Africa, southern Florida, South America: Mexico, Peru, Brazil, and Paraguay are where the *Moringa* plant is grown. It thrives in all soil types and is cultivated in hedges and gardens (Gupta, 2010; Bhattacharjee, 2005).

4. Taxonomical classification

M. oleifera has the following taxonomical hierarchy from kingdom to species:
Kingdom - Plantae
Sub-kingdom - Tracheobionta
Super division - Spermatophyta
Division - Magnoliophyta
Class - Magnoliopsida
Sub-class - Dilleniidae
Order - Capparales
Family - Moringaceae
Genus - *Moringa*
Species - *oleifera* (Fahey, 2005).

5. Common name

The *M. oleifera* plant is known by several names in different languages throughout the world, as presented in Table 1.

6. Botany/Morphology

M. oleifera is a rapidly growing herbaceous tree with a trunk diameter of 20-60 cm and a height of 7-12 m. It has spreading, delicate branches, feathery foliage with tripinnate leaves, and whitish-gray bark (Bashir et al., 2016). Major plant parts encompass the leaves, stem, flowers, fruits, seeds, roots, and bark.

Leaves: The leaves are bipinnate or tripinnate and can grow to be 45 cm long. These are compound leaves with 1-2 cm long leaflets. The upper surface of the leaflets is hairy and green (Paikra and Gidwani, 2017).

Stem: The stem is fragile, with a corky, whitish-gray bark, drooping branches, pale green leaves (30-60 cm long) with opposing, elliptical leaflets, and bipinnate or tripinnate leaves (Pandey et al., 2011).

Flowers: The bisexual fragrance flowers are yellowish-white and borne on long stalks of axillary panicles that spread or droop (10-25 cm long). Individual flowers are 2 cm wide and 0.7 to 1 cm long with

five unequal yellowish-white, spathulate petals with thin veins, five stamens with five smaller sterile filaments, and a pistil with a 1-celled ovary and slender style (Bashir et al., 2016).

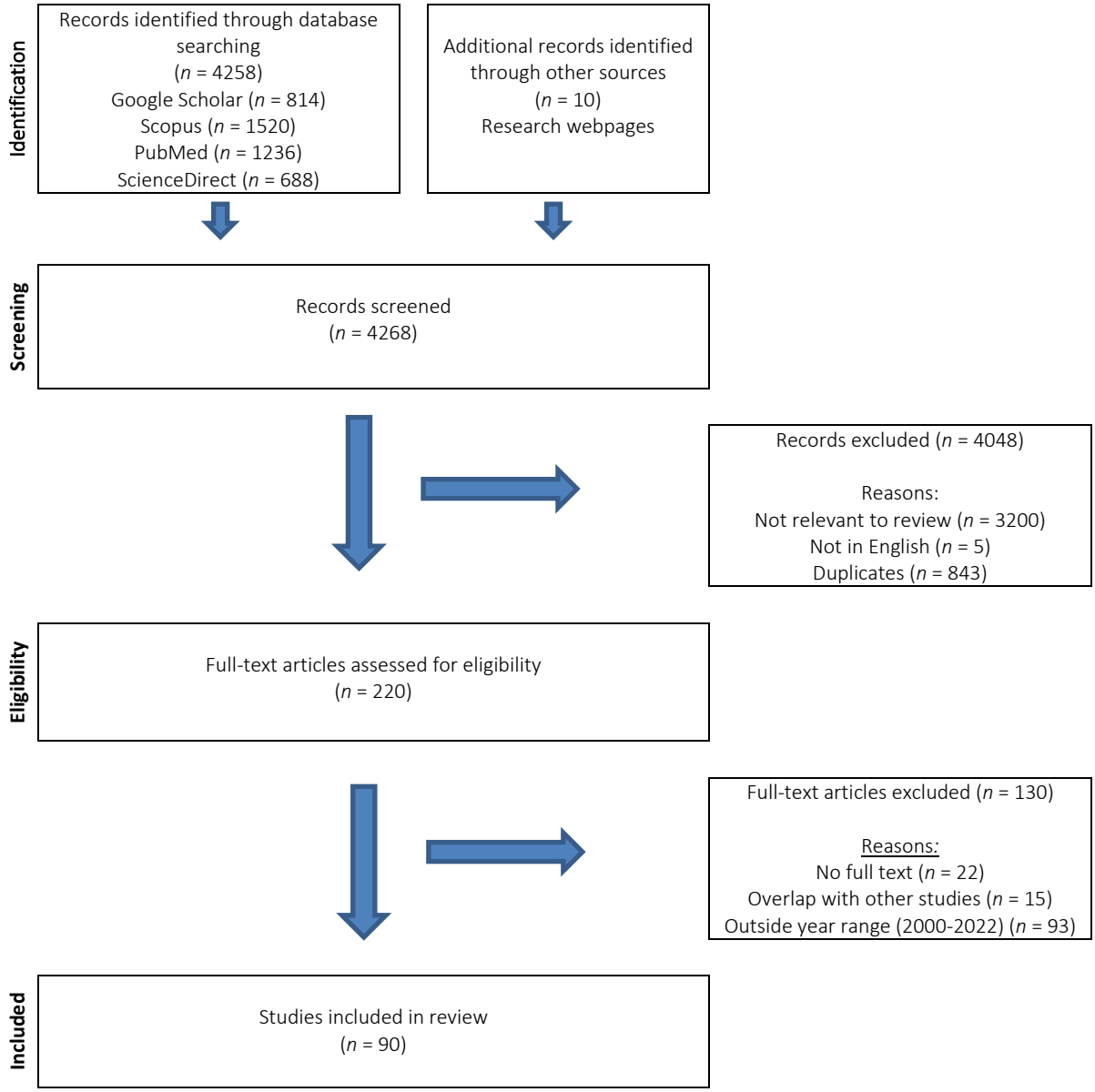


Figure 1. PRISMA flowchart for the review methodology

Table 1. Common names for *M. oleifera* in different languages (Paikra and Gidwani, 2017; Abdull Razis et al., 2014)

Language	Name
English	Drumstick tree, Horseradish tree
French	Benzolive, Bèn ailé
Spanish	Morango, Ben, Moringa
Portuguese	Morungo, Moringuiero
German	Behenbaum, Bennussbaum
Italian	Sandalo ceruleo
Latin	*Moringa oleifera*
Chinese	La ken
Arabian	Rawag
Hindi	Suguna, Sainjna
Malayalam	Murinna, Sigru

Fruits: Fruits are tri-lobed capsules that are commonly known as pods. It is pendulous, brown triangular, and divides into three lengthwise halves when dried. During the development stage of the fruit, it contains approximately 26 seeds. Unripe pods are green and turn brown as they mature (Paikra and Gidwani, 2017).

Seeds: Seeds are 1 cm in diameter, round, with a brownish semi-permeable seed hull and three papery wings. Seed hulls are brown to black but can be white if the kernels are not viable. Within two weeks, viable seeds germinate. Each tree can produce 15.000 to

25.000 seeds per year. The average seed weight is 0.3 grams (Paikra and Gidwani, 2017).

Bark and wood: The bark is whitish gray, thick and soft, fissured and warty or corky, and becomes rough as it ages (Mishra et al., 2020).

Roots: Seedlings have a swollen, tuberous, white taproot with a distinctive pungent odor and very few lateral roots (Mishra et al., 2020). Plates of the various parts of the *M. oleifera* are shown in Figure 2.

7. Phytochemistry

Phytochemicals are chemical elements derived from plants. They are non-nutritive plant chemicals with protective or disease preventive properties (Abd Rani et al., 2018). These chemicals are established to impact human health and contribute to the flavor, texture, smell, and color of plants (Anwar and Bhanger, 2003). *M. oleifera* is rich in a combination of phytochemicals such as terpenes, quercetin, β-sitosterol, caffeoylquinic acid, kaempferol, kaempferitrin, isoquercitrin, rhamnetin, rhamnose, and a fairly unique group of compounds called the glucosinolates and isothiocyanates (Fahey, 2005; Amaglo et al., 2010). Different parts of the *M. oleifera* contain numerous phytochemical compounds. Coppin et al. (2013) reported that the stem bark contains two alkaloids, moringine, and moringinine, with vanillin, β-sitosterol, 4-hydroxymellin, and octacosanoic acid identified in the stem of the plant (Anwar and Bhanger, 2003). The flowers of *M. oleifera* have also been established to contain quercetin and kaempferol (Siddhuraju and Becker, 2003). Studies have also shown the presence of polyphenols, niazimicin, benzyl isothiocyanate, 3-caffeoylquinic, and 5-caffeoylquinic acid in the leaves of *M. oleifera* (Muhammad et al., 2016). A study by Kasolo et al. (2010) identified the presence of protective phytochemicals, including gallic tannins, catechol tannins, steroids and triterpenoids, saponins, anthraquinones, alkaloids, and reducing sugars in ether, ethanol, and aqueous extracts of the roots. The diversity of these phytochemicals in the various parts of the *Moringa* tree contributes to its numerous pharmacological uses (Abd Rani et al., 2018). The chemical structures of selected phytochemical compounds in the *M. oleifera* are shown in Figure 3, with their therapeutic properties presented in Table 2.

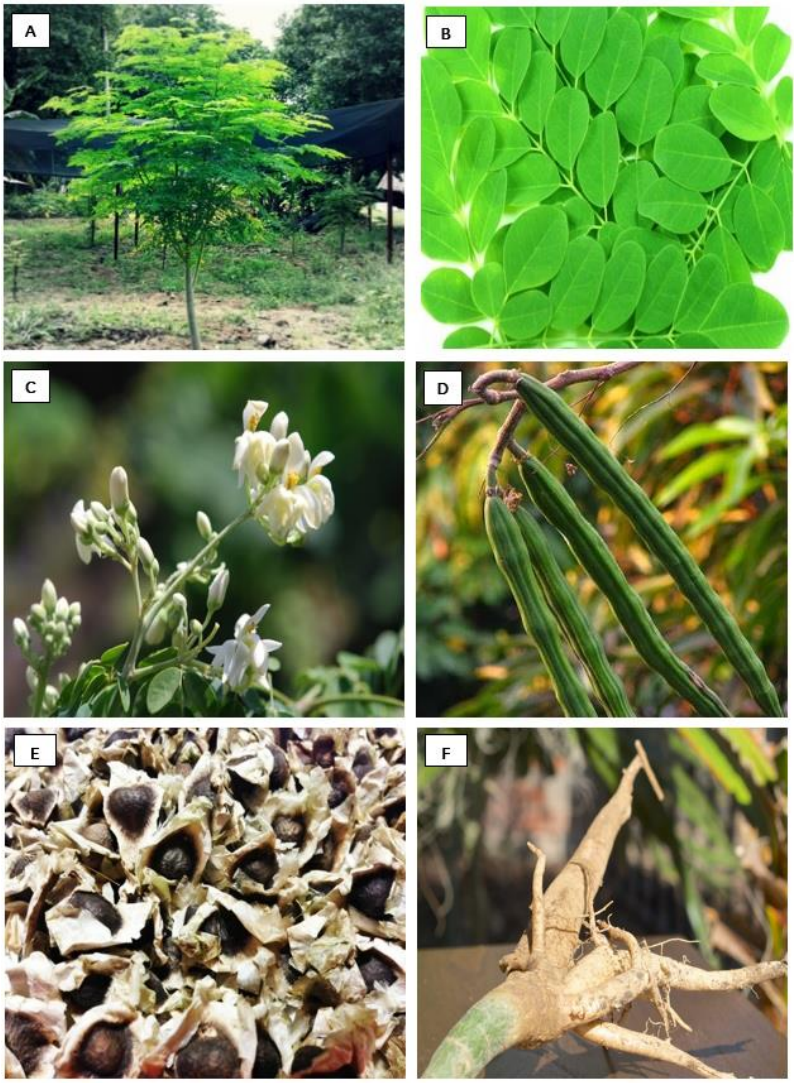
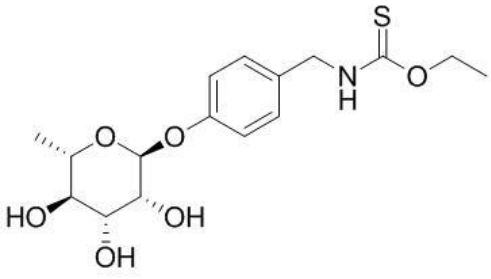


Figure 2. Parts of *M. oleifera* tree
Field grew whole tree (A); Leaves (B); Flowers (C); Fruits (D); Seeds (E); Roots (F)

8. Nutritional composition

There is a wealth of scientific evidence on the nutritional content of *M. oleifera*, which can be linked to its nutritive and medicinal benefits. According to Al-Kharusi et al. (2009) and Abdull Razis et al. (2014), the nutritional value of a plant plays a vital role in its medicinal and therapeutic properties. *M. oleifera* has been found to contain many essential nutrients, including vitamins, minerals, amino acids, beta-carotene, fiber, antioxidants, omega 3 and 6 fatty acids (Kasolo et al., 2010; Fahey, 2005). Fahey (2005) reported that *Moringa* leaves have a higher vitamin C content than oranges, a higher vitamin A content than carrots, a higher calcium content than milk, a higher iron content than spinach, and higher potassium content than bananas and that the protein quality of the leaves is comparable to that of milk and eggs. *M. oleifera* immature pods also contain approximately 46.78 percent fiber, 20.66 percent protein, and 30 percent of amino acid (Mishra et al., 2020). Many parts of the plant serve as a repository for these vital nutrients. As a result, it is unusual for a single plant to contain several essential nutrients in such high concentrations as *M. oleifera*.



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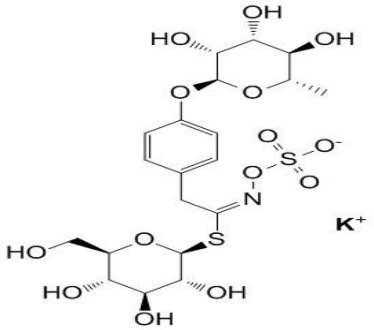

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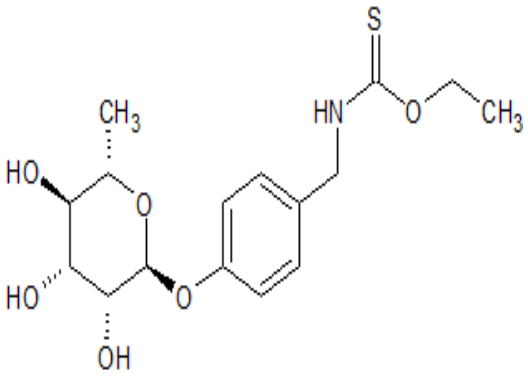

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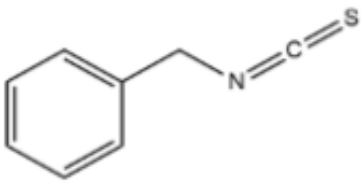

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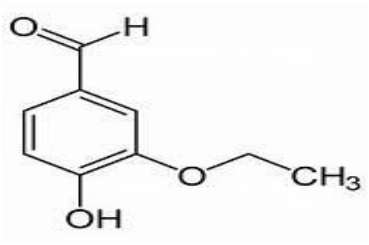

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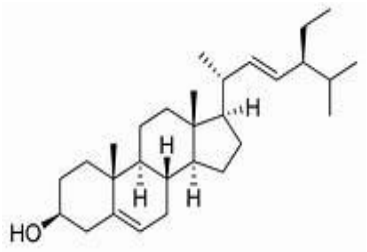

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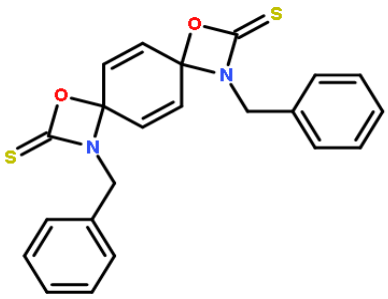

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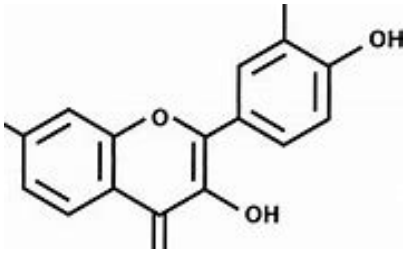

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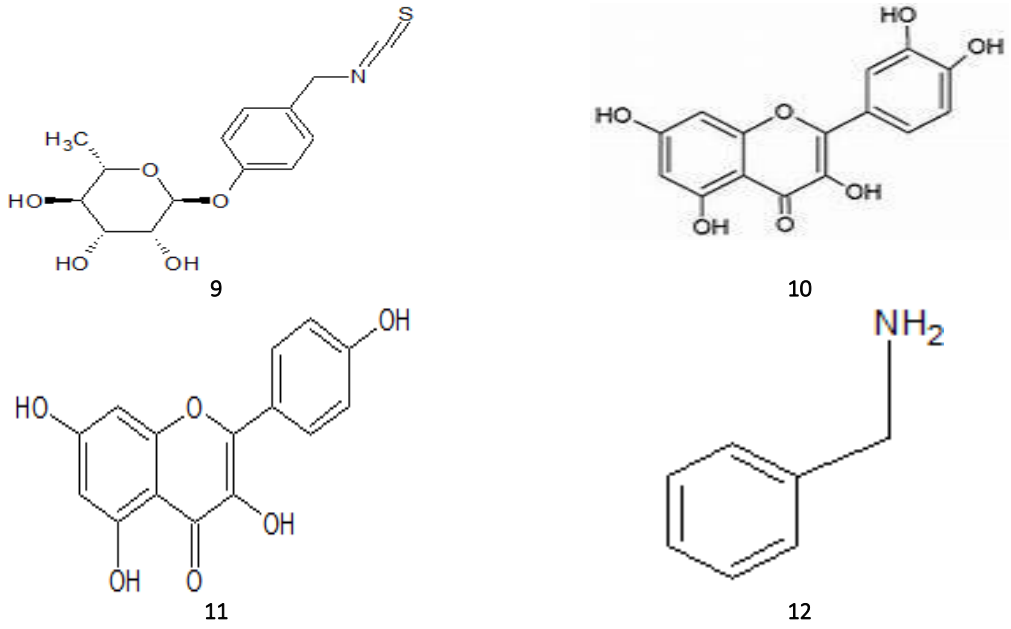

Figure 3. Structures of selected phytochemicals isolated from *M. oleifera*
Glucosinolate (1), Glucomoringin (2), Niazimicin (3), Benzyl isothiocyanate (4), Vanillin (5), β-sitosterone (6), Pterygospermin (7), Terpenoid (8) 4-(α-L-rhamnosyloxy) benzyl isothiocyanate (9), Quercetin (10), Kaempferol (11), Moriginine (12)

Vitamins are one of the abundant nutrients, particularly in the leaves of *M. oleifera*. Dillard and German (2000), as well as Toma and Deyno (2014) stated that *M. oleifera* leaves are rich in vitamin C, β-carotene, protein, calcium, and potassium and are excellent sources of natural antioxidants. Mishra et al. (2020) also reported that *M. oleifera* leaves contain vitamins including vitamin B such as folic acid, beta-carotene of vitamin A, vitamin C, D, and E, pyridoxine, and nicotinic acid. Fresh leaves have also been proven to have higher levels of vitamin C than typical sources like oranges (Leone et al., 2015). A high content like this is significant due to vitamin C's role in facilitating the conversion of cholesterol into bile acids, thereby lowering blood cholesterol levels (Leone et al., 2015). Vitamin E, particularly α-tocopherol, is also established in the leaves of *M. oleifera* in amounts comparable to those found in the nuts (Sánchez-Machado et al., 2006).

Table 2. List of selected phytochemicals in *M. oleifera* and their biological properties

No	Phytochemical	Plant part	Biological property	Reference
1	Glucosinolate	Leaves	Chemopreventive activity by activating apoptosis	Waterman et al., 2014
2	Glucomoringin	Leaves	Anti-colon, carcinogenic activity	Fahey et al., 2018
3	Niazimicin	Leaves	Anti-cancer activity	Adejumo et al., 2012
4	Benzyl isothiocyanate	Leaves	Anti-inflammatory and anti-cancer activity	Waterman et al., 2014
5	Vanillin	Stem	Anti-cancer, neuroprotective activity	Bennett et al., 2003
6	β-sitosterone	Stem	Antioxidant, hypolipidemic activity	Rajanandh and Kavitha, 2010
7	Pterygospermin	Seeds	Anti-herpes, cramps, rheumatism, epilepsy	Biswas et al., 2020
8	Terpenoid	Seeds	Anti-cancer, anti-hyperglycemic, and antibacterial activity	Mehra et al., 2017; Huang et al., 2012
9	4-(α-L-rhamnosyloxy) benzyl isothiocyanate	Seeds	Antimicrobial, antitumor enhancer	Popoola et al., 2020
10	Quercetin	Flowers	Inhibit mutations, prevent carcinogenesis	Imran et al., 2019; Leone et al., 2015
11	Kaempferol	Flowers	Antioxidant, inhibit mutations	Imran et al., 2019; El-Alfy et al., 2011
12	Moriginine	Bark, stem	Anti-inflammatory and anti-ulcer activity	Rajbhar et al., 2018

Vitamin E acts principally as an antioxidant agent, but it is also involved in gene expression regulation, inhibition of cell proliferation, platelet aggregation, monocyte adhesion, and bone mass regulation (Sánchez-Machado et al., 2006). A few B vitamins, comprising riboflavin, thiamine, and niacin, have also been found in the leaves of *M. oleifera* (Leone et al., 2015). These vitamins primarily function as co-factors for numerous enzymes involved in nutrient metabolism and energy production (Leone et al., 2015).

Moringa contains a diversity of minerals necessary for healthy growth and development. The most abundant minerals in the tissues of *M. oleifera* are calcium (Ca), potassium (K), and magnesium (Mg) (Amaglo et al., 2010). The immature pods and vegetative parts have the maximum K, whereas the seeds and leaves contain more Mg and Ca (Amaglo et al., 2010). *M. oleifera*'s leaves are also a superb source of iron, zinc, and copper (Saini et al., 2016; Mishra et al., 2020). *M. oleifera* leaves contain approximately 25.5 to 31.03 mg/kg of zinc, the daily zinc requirement in diet (Gopalakrishnan et al., 2016). Zinc is required for the normal proliferation of sperm cells as well as the synthesis of DNA and RNA (Gopalakrishnan et al., 2016). Regarding iron, Fuglie (2005) reported that *Moringa* contains more iron than spinach. He also added that moringa leaves contain more iron than beef. Thus, beef contains only 2 mg of iron, whereas *Moringa* leaf powder contains 28 mg of iron. Hence, *Moringa* powder can be used as a substitute for iron tablets to treat anemia.

Fatty acids have also been found in some parts of the *Moringa* tree. Saini et al. (2016) stated that the *Moringa* leaves contain omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) in the form of linolenic acid and linoleic acid. When equated to the leaves, unripe pods and flowers have a higher concentration of total monounsaturated fatty acids (MUFAs, 16-30%) and a minor content of polyunsaturated fatty acids (PUFAs, 34-47%) (Saini et al., 2016).

Moreover, palmitic acid has also been identified as the most copious saturated fatty acid in *Moringa* leaves, accounting for 16-18% of the overall fatty acids (Saini et al., 2016). Studies have as well shown that the seed oil of *Moringa* contains approximately 76 percent PUFA, making it an excellent olive oil substitute (Lalas and Tsaknis, 2002). These PUFAs can lower cholesterol (Lalas and Tsaknis, 2002). The *Moringa* seeds and seed oil also contain substantial amounts of oleic, stearic, arachidic acid, linoleic, and linolenic acids (Amaglo et al., 2010).

9. Pharmacological studies

Some scientific investigations have reported that specific components of *M. oleifera* preparations possess many pharmacological actions, including antimicrobial, anti-inflammatory, anticancer and antitumor, antidiabetic, antifertility, antioxidant, anti-asthmatic, hepatoprotective, cardiovascular, and immunomodulatory activities. Key reports and findings on these important pharmacological properties ascribed to *M. oleifera* are compiled below.

9.1. Antibacterial and antifungal

Phytochemicals and plant extracts with antimicrobial properties are important in therapeutics (Moyo et al., 2012). Due to antimicrobial resistance, there has been a surge in interest in medicinal plants possessing such capabilities. *M. oleifera* is an effective antibacterial agent, as evidenced by studies showing a significant reduction in the growth of test bacteria. In a study using erythromycin as a positive control, Gopalakrishnan et al. (2016) discovered that *M. oleifera* had potential antibacterial activity against four key bacterial strains tested: *Staphylococcus aureus*, *Bacillus megaterium*, *Citrobacter freundii*, and *Pseudomonas fluorescens*. Vinoth et al. (2012) also conducted a study on *M. oleifera* where the aqueous and ethanolic leaf extracts demonstrated antibacterial activity against *Salmonella* spp., while acetone leaf extracts at a concentration of 5 mg/mL also displayed antibacterial activity against *S. aureus*, *Escherichia coli*, *Enterobacter cloacae*, *Proteus vulgaris*, and *Micrococcus kristinae*. The bark extract of the *Moringa* tree has also been demonstrated to possess antifungal properties (Toma and Deyno, 2014). Ringworms are mostly treated with *M. oleifera* seed oil, according to Shikwambana and Mahlo (2020). Isitua et al. (2016) stated that *M. oleifera* leaves extract could potentially be used to treat aspergillosis in humans, based on their findings, which showed high antifungal activity against *Aspergillus flavus*, a fungus that causes aspergillosis in humans. Kou et al. (2018) also found that the aglycone of deoxy-niazimicin extracted from the chloroform portion of ethanol extract was accountable for the antifungal and antibacterial activity in the root bark of *M. oleifera*. These findings and reports prove the ability of *M. oleifera* extracts to inhibit the growth of some strains of bacteria and fungi, indicating their potential to either block or outwit resistance mechanisms by these pathogenic microorganisms.

9.2. Anti-inflammatory activity

Inflammation is a frequent symptom of a diverse array of chronic infections. Anti-inflammatory mediators are in charge of alleviating pain and reducing inflammation, thereby promoting health. Because of their safety and effectiveness, medicinal plants as anti-inflammatory agents are considered viable and logical options (Alhakmani et al., 2013). Several studies have been conducted to investigate *M. oleifera*'s anti-inflammatory properties. Ndiaye et al. (2002) reported that the aqueous extract of the root of *M. oleifera* (750 mg/kg) displays anti-inflammatory activity by obstructing carrageenan-induced edema in rats to the same level as the effective anti-inflammatory drug indomethacin. A study by Fidrianny et al. (2021) also inferred that the anti-inflammatory properties of silver nanoparticles (AgNps) synthesized from *M. oleifera* flowers exhibited a significant and higher inhibition percentage by albumin denaturation activity. *Moringa* seed extract's hepatoprotective properties, discovered in an anti-fibrotic study by Hamza (2010), indicated that *Moringa* possesses anti-inflammatory properties against CCL_4-induced liver damage and fibrosis.

9.3. Anticancer and antitumor activity

On a global scale, cancer is responsible for around 1 in every 6 mortalities (Singh et al., 2020). Plants are frequently used to produce clinically relevant antitumor chemicals that can be turned into anticancer drugs (Kamuhabwa et al., 2000). One of the numerous benefits of employing dietary or natural chemicals as a cancer adjuvant is that they are low in toxicity and have few negative side effects (Nair and Varalakshmi, 2011). *M. oleifera* has been shown to have anticancer effects in several investigations. In chemical carcinogenesis, niazimicin, a chemical molecule found in *M. oleifera*, has been postulated as a strong chemopreventive agent (Toma and Deyno, 2014). Hagoel et al. (2019) reported in a study that the aqueous leaf extract of *M. oleifera* lessens pancreatic cancer cell existence, tumor growth, and metastatic actions. Anticancer drugs typically target ROS induction. However, *Moringa* leaf extracts were discovered to have the ability to attack ROS induction (Gopalakrishnan et al., 2016). Earlier research has demonstrated that intake of *M. oleifera* seedpod extracts can prevent skin tumors, including a significant reduction in skin papillomas in a mouse model (Bharali et al., 2003). Toma and Deyno (2014) also found that niaziminin, a thiocarbamate derived from the leaves of *M. oleifera*, inhibits tumor-promoter-induced Epstein–Barr virus activation.

9.4. Antioxidant activity

Antioxidants are chemicals that provide free atoms to the human body while suppressing free radicals, which cause cell damage and oxidative stress. Natural antioxidants are abundant in medicinal plants. *Moringa* leaves have been established to be high in natural antioxidants such as flavonoids, quercetin, β-sitosterol, and zeatin (Fahey, 2005; Amaglo et al., 2010). Tender and mature leaf extracts were found to have great scavenging properties on free radicals, nitric oxide radicals, and superoxides (Sreelatha and Padma, 2009). Ethanol, methanol, and water extracts of freeze-dried *Moringa* leaves were also found to have antioxidant and free radical scavenging properties, with all leaf extracts capable of scavenging superoxyl and peroxyl radicals, according to Siddhuraju and Becker (2003). *M. oleifera* also showed excellent antioxidant activity from the most isolated compounds in different *in vitro* bioassays compared to typical antioxidant compounds in a study that employed the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical technique (Hamed et al., 2017). Furthermore, an *in vivo* study conducted by Jaja-Chimedza et al. (2018) on normal and obese male mice demonstrated that *Moringa* seed extract could increase metabolic health through its intracellular antioxidant and anti-inflammatory actions.

9.5. Antidiabetic activity

Diabetes is one of the most common metabolic illnesses in the world, and it is a huge public health issue. Hyperglycemia is usually treated with synthetic oral medications and insulin remedies. However, many of these medications have negative side effects and are too expensive for disadvantaged people, particularly in emerging

nations. As a result, the demand for cost-effective and efficient hypoglycemic medicines with fewer side effects will always exist. *Moringa* has been demonstrated to have anti-diabetic properties in several investigations. Muhammad et al. (2016) investigated the anti-diabetic effect of *M. oleifera* seeds, roots, and stem bark extracts in diabetic mice and reported that the aqueous, ethanolic, and methanolic extracts of these plant parts provided good glycemic control in the diabetic animals. Another study by Irfan et al. (2017) established that the anti-hyperglycemic activity in diabetic rats was due to the kaempferol-3-*O*-glucoside, quercetin-3-D-glucoside, and crypto chlorogenic acid in the leaf extract of *M. oleifera*. In streptozotocin (STZ)-induced diabetic albino rats, Gupta et al. (2012) found that the methanolic pods extract of *M. oleifera* has anti-diabetic properties. *Moringa* seed powder effectively lowered fasting blood sugar in STZ-induced diabetic rats in a similar study by Owens et al. (2020).

9.6. Anti-asthmatic activity

Asthma is a long-lasting respiratory disease that affects a considerable part of the world's population, accounting for about 90% of cases in most global surveys (Mahajan et al., 2009). For hundreds of years, complementary and alternative medicine has been utilized to treat asthma. *M. oleifera*, in particular, is effective in treating asthma, with literature describing the plant's anti-asthmatic properties. In guinea pigs, Mahajan et al. (2009) tested the efficiency of *n*-butanol extracts of *M. oleifera* seeds against ovalbumin-induced airway inflammation. The study found that the seeds extract of *M. oleifera* seed extracts protect against acetylcholine-induced bronchoconstriction and airway inflammation by increasing tidal volume and respiration rate, as well as differential and total cell counts in blood and bronchoalveolar lavage fluid. They concluded that the anti-asthmatic properties of *M. oleifera* seed extracts were due to the regulation of Th1/Th2 cytokine imbalances. In another study by Singh and Navneet (2018), *M. oleifera* seed kernels used in bronchial asthma demonstrated a simultaneous improvement in respiratory functions and a significant reduction in the severity of asthma symptoms. Agrawal and Mehta (2008) also conducted a clinical trial to evaluate if *M. oleifera* seed kernels effectively treat bronchial asthma. The dried seed kernels were given to 20 patients with mild to moderate asthma at a dose of 3 kg for three weeks. The results showed that treatment with *M. oleifera* extracts resulted in a significant reduction in the intensity of asthma symptoms as well as an improvement in lung function metrics.

9.7. Antifertility activity

Women have traditionally utilized medicinal plants to aid childbirth, promote menstrual flow, and limit fertility (Agrawal et al., 2018). *M. oleifera* is well-known for its therapeutic benefits. However, its antifertility properties have also been studied. In female albino rats, Zade and Dabhadkar (2015) found that alcoholic extracts of *M. oleifera* leaves had an antifertility effect. In that study, *M. oleifera* leaf extract was found to prevent 100% of implantation in seven rats. Mishra et al. (2011) also studied the antifertility properties of *M. oleifera* aqueous and ethanolic (90%) extracts in rats orally dosed for 10 days after insemination, with emphasis on fetal development. *M. oleifera* leaf extracts were completely (100%) abortive at dosages of 175 mg/kg of initial dry material. *M. oleifera*'s antifertility properties and prospects could make it a valuable option for modern contraception and antifertility issues for women, especially in rural populations in underdeveloped nations with limited financial resources and access to healthcare.

9.8. Hepatoprotective activity

The liver is an important organ that conducts various activities, including detoxification and protein synthesis. The effects can be fatal when the liver's activities are harmed by the many substances to which humans are exposed. Many medications used to treat liver problems are insufficient and may pose dangerous side effects. The search for efficient hepatoprotective medications frequently focuses on medicinal plants used to treat some liver diseases (Buraimoh et al., 2011). Tejas et al. (2012) reported that the methanolic leaf extract of *M. oleifera* has a hepatoprotective effect, which could be attributed to the presence of quercetin in the leaves. Buraimoh et al. (2011) also investigated the hepatoprotective effects of *M. oleifera* ethanolic leaf extract on paracetamol-induced liver damage histopathology. Compared to the negative control group, which had deformed hepatic cords, necrotic cells, and obliterated sinusoids, Wistar rats given extracts (500 mg/kg) had less necrotic cell damage and broader sinusoidal gaps. *M. oleifera* leaves have also been demonstrated to lower plasma aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and creatinine (Sharifudin et al., 2013), as well as alleviate hepatic and renal damage caused by medications (Ouédraogo et al., 2013). *Moringa* flower extracts, both aqueous and alcoholic, have also been demonstrated to possess strong hepatoprotective properties (Toma and Deyno, 2014).

9.9. Cardiovascular activity

A multitude of ailments that affect the circulatory system (heart and blood vessels) is cardiovascular disease. For millennia, medicinal plants have been utilized to treat cardiovascular disorders. This is due to their antioxidant, vasodilator, adrenoceptor, and platelet-activating factor (PAF) antagonist properties (Chumark et al., 2008). The cardiovascular effects of *M. oleifera* have been investigated using animal and human models. Randriamboavonjy et al. (2016) studied the heart effects of *M. oleifera* seed powder given orally to spontaneously hypertensive rats (SHR). SHR was given either *Moringa* powdered food (750 mg/day for 8 weeks) or regular food. After that, hemodynamic parameters were measured *in vivo*. According to the findings, *M. oleifera* treatments did not change blood pressure in SHR but did lower nocturnal heart rate and improved diastolic cardiac function. Nandave et al. (2009) investigated the cardioprotective efficacy of lyophilized hydroalcoholic extract of *M. oleifera* in a male Wistar albino rat model of myocardial infarction induced by isoproterenol (ISP). Compared to the ISP control group, chronic *Moringa* therapy resulted in a significant increase in the positive regulation of biological enzymes (catalase, superoxide dismutase, lactate dehydrogenase, creatine kinase, and glutathione peroxidase), but no significant change in reduced glutathione. *Moringa* treatment significantly reduced lipid peroxidation in heart tissue. According to Chhikara et al. (2020), oxidative stress-induced chronic diseases such as cardiovascular issues could be reduced due to flavonoids in *M. oleifera*.

9.10. Immunomodulatory activity

Plant-based immunomodulation is a hot topic in scientific circles since it offers a viable alternative to traditional chemotherapy for various disorders. It is based on plants' ability to control immunological activities efficiently, promoting positive health and maintaining the body's resistance to illness. Sudha et al. (2010) investigated the immunomodulatory effects of a methanolic extract of *M. oleifera* leaves in a mouse cellular and humoral immunity model. The extracts were given at two doses (250 and 750 mg/kg) to

mice, which significantly boosted serum immunoglobulin levels and avoided mortality caused by bovine *Pasteurella multocida*. Alkaloids are abundant phytochemicals in *M. oleifera* and have immunomodulatory properties, according to Obi et al. (2018). Certain bitter alkaloids, such as tropane alkaloids, are converted into dimethylxanthine in the liver and then to methyl uric via CYP450 oxygenase systems (Obi et al., 2018). Gupta (2010) also found that ethanolic leaf extracts of *M. oleifera* have an immunomodulatory effect in mice, leading cyclophosphamide-induced immunosuppression to be significantly reduced. After prolonged administration of the extracts, normal mice showed a considerable rise in white blood cell (WBC) count, phagocytic index, percent neutrophils, and thymus and spleen weight.

10. Future prospects

M. oleifera, with its rich source of nutrients, abundant phytochemicals, and numerous pharmacological actions, has the potency to help combat malnutrition and improve medicine. The diverse nutritional elements of the plant, particularly in the leaves, have a promising role in producing healthy food products and nutraceuticals. The abundant phytochemical compounds in distinct parts of the plant have the potential to be used in the manufacturing of pharmaceutical products and offer therapies for a wide range of diseases. Industrially, the plant also has a fascinating prospect of being used in the production of biodiesel (the seeds), cosmetics, and water purification systems. Hence, *M. oleifera* deserves extensive investigations to isolate and better elucidate the mechanism of actions of the various active compounds responsible for its nutritional and medicinal efficacies.

11. Conclusions

M. oleifera, with its unique nutritive and medicinal properties, has a remarkable potential to provide an inexpensive and credible auxiliary to conventional medicine. The present review documented the phytochemical compounds, nutritional composition, and pharmacological potential of *M. oleifera*. Numerous scientific investigations revealed that individual parts of the plant possess anticancer, antimicrobial, anti-inflammatory, antidiabetic, antifertility, hepatoprotective, cardiovascular, and immunomodulatory actions. Many essential nutrients, including vitamins, minerals, proteins, beta-carotene, fiber, and fatty acids, were also present in *M. oleifera*, justifying the plant's usage for decades to combat malnutrition.

Moreover, medicinally important phytochemicals such as alkaloids, flavonoids, terpenes, sterols, flavonoids, glycosides, and anthocyanins were established to be present adequately in *M. oleifera*; and this makes the plant therapeutically imperative and/or nutritionally valuable. The present review recommends in-depth investigations into the isolation and purification of novel pharmacologically active and industrially important phytochemical compounds in *M. oleifera*, as much work has not been conducted in this field. Additionally, more rigorous research on the toxicity of *M. oleifera* is required, including clinical trials in humans, to ascertain its safety and achieve a level of proof required for full biomedical endorsement of the plant for therapeutics.

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Conflict of interest

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Supplementary File

None.

References

Abd Rani, N.Z., Husain, K., Kumolosasi, E., 2018. *Moringa* genus: a review of phytochemistry and pharmacology. *Frontiers in Pharmacology*, 9, 108.
Abdull Razis, A.F., Ibrahim, M.D., Kntayya, S.B., 2014. Health benefits of *Moringa oleifera*. *Asian Pacific Journal of Cancer Prevention*, 15(20), 8571-8576.
Adejumo, O.E., Kolapo, A.L., Folarin, A.O., 2012. *Moringa oleifera* Lam.(Moringaceae) grown in Nigeria: *In vitro* antisickling activity on deoxygenated erythrocyte cells. *Journal of Pharmacy & Bioallied Sciences*, 4(2), 118-122.
Agrawal, B., Mehta, A., 2008. Antiasthmatic activity of *Moringa oleifera* Lam: A clinical study. *Indian Journal of Pharmacology*, 40(1), 28-31.
Agrawal, S.S., Vishal, D., Sumeet, G., Shekhar, C., Ashish, N., Parul, D., Varun, S., 2018. Antifertility activity of ethanol leaf extract of *Moringa oleifera* Lam in female wistar rats. *Indian Journal of Pharmaceutical Sciences*, 80(3), 565-570.
Alhakmani, F., Kumar, S., Khan, S.A., 2013. Estimation of total phenolic content, *in–vitro* antioxidant and anti–inflammatory activity of flowers of *Moringa oleifera*. *Asian Pacific Journal of Tropical Biomedicine*, 3(8), 623-627.
Al-Kharusi, L.M., Elmardi, M.O., Ali, A., Al-Said, F.A.J., Abdelbasit, K.M., Al-Rawahi, S., 2009. Effect of mineral and organic fertilizers on the chemical characteristics and quality of date fruits. *International Journal of Agriculture and Biology*, 11(3), 290-296.
Amaglo, N.K., Bennett, R.N., Curto, R.B.L., Rosa, E.A., Turco, V.L., Giuffrida, A., Timpo, G.M., 2010. Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L., grown in Ghana. *Food Chemistry*, 122(4), 1047-1054.
Anwar, F., Bhanger, M.I., 2003. Analytical characterization of *Moringa oleifera* seed oil grown in temperate regions of Pakistan. *Journal of Agricultural and Food Chemistry*, 51(22), 6558-6563.
Azam, M.M., Waris, A., Nahar, N.M., 2005. Prospects and potential of fatty acid methyl esters of some non-traditional seed oils for use as biodiesel in India. *Biomass and Bioenergy*, 29(4), 293-302.
Bashir, K.A., Waziri, A.F., Musa, D.D., 2016. *Moringa oleifera*, a potential miracle tree; a review. *IOSR Journal of Pharmacy and Biological Sciences*, 11, 25-30.
Bennett, R.N., Mellon, F.A., Foidl, N., Pratt, J.H., Dupont, M.S., Perkins, L., Kroon, P.A., 2003. Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L.(horseradish tree) and *Moringa stenopetala* L. *Journal of Agricultural and Food Chemistry*, 51(12), 3546-3553.
Berushka, P., Himansu, B., 2012. An overview of the medicinal importance of Moringaceae. *Journal of Medicinal Plants Research*, 6(48), 5831-5839.
Bharali, R., Tabassum, J., Azad, M.R.H., 2003. Chemomodulatory effect of *Moringa oleifera*, Lam, on hepatic carcinogen metabolising enzymes, antioxidant parameters and skin papillomagenesis in mice. *Asian Pacific Journal of Cancer Prevention*, 4(2), 131-140.
Bhattacharjee, K.S.,2005. Hand Book of Aromatic Plants. 2nd Revised and Enlarged Edition. Pointer Publishers, India, 298.

Biswas, D., Nandy, S., Mukherjee, A., Pandey, D.K., Dey, A., 2020. *Moringa oleifera* Lam. and derived phytochemicals as promising antiviral agents: A review. *South African Journal of Botany*, 129, 272-282.

Buraimoh, A.A., Bako, I.G., Ibrahim, F.B., 2011. Hepatoprotective effect of ethanolic leave extract of *Moringa oleifera* on the histology of paracetamol induced liver damage in Wistar rats. *International Journal of Animal and Veterinary Advances*, 3(1), 10-13.

Chhikara, N., Kaur, A., Mann, S., Garg, M.K., Sofi, S.A., Panghal, A., 2020. Bioactive compounds, associated health benefits and safety considerations of *Moringa oleifera* L.: An updated review. *Nutrition & Food Science*, 51(2), 255-277.

Chumark, P., Khunawat, P., Sanvarinda, Y., Phornchirasilp, S., Morales, N.P., Phivthong-Ngam, L., Klai-upsorn, S.P., 2008. The *in vitro* and *ex vivo* antioxidant properties, hypolipidaemic and antiatherosclerotic activities of water extract of *Moringa oleifera* Lam. leaves. *Journal of Ethnopharmacology*, 116(3), 439-446.

Coppin, J.P., Xu, Y., Chen, H., Pan, M.H., Ho, C.T., Juliani, R., Wu, Q., 2013. Determination of flavonoids by LC/MS and anti-inflammatory activity in *Moringa oleifera*. *Journal of Functional Foods*, 5(4), 1892-1899.

Dillard, C.J., German, J.B., 2000. Phytochemicals: nutraceuticals and human health. *Journal of the Science of Food and Agriculture*, 80(12), 1744-1756.

Efiong, E.E., Igile, G.O., Mgbeje, B.I.A., Otu, E.A., Ebong, P.E., 2013. Hepatoprotective and anti-diabetic effect of combined extracts of *Moringa oleifera* and *Vernonia amygdalina* in streptozotocin-induced diabetic albino Wistar rats. *Journal of Diabetes and Endocrinology*, 4(4), 45-50.

Ekor, M., 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, 4, 177.

El-Alfy, T.S., Ezzat, S.M., Hegazy, A.K., Amer, A.M., Kamel, G.M., 2011. Isolation of biologically active constituents from *Moringa peregrina* (Forssk.) Fiori.(family: Moringaceae) growing in Egypt. *Pharmacognosy Magazine*, 7(26), 109-115.

Fahey, J.W., 2005. *Moringa oleifera*: a review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. *Trees for Life Journal*, 1(5), 1-15.

Fahey, J.W., Olson, M.E., Stephenson, K.K., Wade, K.L., Chodur, G.M., Odee, D., Hubbard, W.C., 2018. The diversity of chemoprotective glucosinolates in Moringaceae (*Moringa* spp.). *Scientific Reports*, 8(1), 1-14.

Fidrianny, I., Kanapa, I., Singgih, M., 2021. Phytochemistry and pharmacology of moringa tree: an overview. *Biointerface Research in Applied Chemistry*, 11(3), 10776-10789.

Fuglie, L.J., 2005. The *Moringa* Tree. A local solution to malnutrition, http://www.moringa news.org/documents/Nutrition, (Accessed:15 March, 2022).

Gopalakrishnan, L., Doriya, K., Kumar, D.S., 2016. *Moringa oleifera*: A review on nutritive importance and its medicinal application. *Food Science and Human Wellness*, 5(2), 49-56.

Gupta, R., Mathur, M., Bajaj, V.K., Katariya, P., Yadav, S., Kamal, R., Gupta, R.S., 2012. Evaluation of antidiabetic and antioxidant activity of *Moringa oleifera* in experimental diabetes. *Journal of Diabetes*, 4(2), 164-171.

Gupta, R.K., 2010. Medicinal and aromatic plants. CBS publishers and distributors, 234, 499.

Hagoel, L., Vexler, A., Kalich-Philosoph, L., Earon, G., Ron, I., Shtabsky, A., Lev-Ari, S., 2019. Combined effect of *Moringa oleifera* and ionizing radiation on survival and metastatic activity of pancreatic cancer cells. *Integrative Cancer Therapies*, 18, 1534735419828829.

Halaby, M.S., Metwally, E.M., Omar, A.A., 2013. Effect of *Moringa oleifera* on serum lipids and kidney function of hyperlipidemic rats. *Journal of Applied Sciences Research*, 9, 5189-5198.

Hamed, M.M., Mohamed, M.A.M., Ahmed, W.S., 2017. Chemical constituents, *in vitro* antioxidant activity, oral acute toxicity and LD_{50} determination of *Moringa oleifera* leaves. *International Journal of Pharmacy and Pharmaceutical Sciences*, 9(5), 240-247.

Hamza, A.A., 2010. Ameliorative effects of *Moringa oleifera* Lam seed extract on liver fibrosis in rats. *Food and Chemical Toxicology*, 48(1), 345-355.

Huang, M., Lu, J.J., Huang, M.Q., Bao, J.L., Chen, X.P., Wang, Y.T., 2012. Terpenoids: natural products for cancer therapy. *Expert Opinion on Investigational Drugs*, 21(12), 1801-1818.

Imran, M., Salehi, B., Sharifi-Rad, J., Aslam Gondal, T., Saeed, F., Imran, A., Estevinho, L.M., 2019. Kaempferol: A key emphasis to its anticancer potential. *Molecules*, 24(12), 2277.

Irfan, H.M., Asmawi, M.Z., Khan, N.A.K., Sadikun, A., Mordi, M.N., 2017. Anti-diabetic activity-guided screening of aqueous-ethanol *Moringa oleifera* extracts and fractions: Identification of marker compounds. *Tropical Journal of Pharmaceutical Research*, 16(3), 543-552.

Isitua, C.C., Ibeh, I.N., Olayinka, J.N., 2016. *In vitro* Antifungal Activity of *Moringa oleifera* Lam Leaf on some selected clinical fungal strains. *Indian Journal of Applied Research*, 6(8), 548-552.

Jaja-Chimedza, A., Zhang, L., Wolff, K., Graf, B.L., Kuhn, P., Moskal, K., Raskin, I., 2018. A dietary isothiocyanate-enriched moringa (*Moringa oleifera*) seed extract improves glucose tolerance in a high-fat-diet mouse model and modulates the gut microbiome. *Journal of Functional Foods*, 47, 376-385.

Kamuhabwa, A., Nshimo, C., de Witte, P., 2000. Cytotoxicity of some medicinal plant extracts used in Tanzanian traditional medicine. *Journal of Ethnopharmacology*, 70(2), 143-149.

Kasolo, J.N., Bimenya, G.S., Ojok, L., Ochieng, J., Ogwal-Okeng, J.W., 2010. Phytochemicals and uses of *Moringa oleifera* leaves in Ugandan rural communities. *Journal of Medicinal Plants Research*, 4(9), 753-757.

Kenwat, R., Prasad, P., Satapathy, T., Roy, A., 2013. *Martynia annua*: an overview. *Pharmaceutical and Biosciences Journal*, 1(1), 7-10.

Kou, X., Li, B., Olayanju, J.B., Drake, J.M., Chen, N., 2018. Nutraceutical or pharmacological potential of *Moringa oleifera* Lam. *Nutrients*, 10(3), 343.

Kumar, S., 2013. Systematic pharmacognostical, phytochemical and pharmacological review on an ethno medicinal plant, *Basella alba* L. *Journal of Pharmacognosy and Phytotherapy*, 5(4), 53-58.

Lalas, S., Tsaknis, J., 2002. Characterization of *Moringa oleifera* seed oil variety "Periyakulam 1". *Journal of Food Composition and Analysis*, 15(1), 65-77.

Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., Bertoli, S., 2015. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: An overview. *International Journal of Molecular Sciences*, 16(6), 12791-12835.

Mahajan, S.G., Banerjee, A., Chauhan, B.F., Padh, H., Nivsarkar, M., Mehta, A.A., 2009. Inhibitory effect of n-butanol fraction of *Moringa oleifera* Lam. seeds on ovalbumin-induced airway inflammation in a guinea pig model of asthma. International Journal of Toxicology, 28(6), 519-527.

Mahmood, K.T., Mugal, T., Haq, I.U., 2010. *Moringa oleifera*: a natural gift-A review. *Journal of Pharmaceutical Sciences and Research*, 2(11), 775.

Mbikay, M., 2012. Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: a review. *Frontiers in Pharmacology*, 3, 24.

Mehra, M., Jakhar, N., Joshi, S., Meghwal, M., 2017. Phytotherapaeutic functionality of *Moringa oleifera* Lam for health. *International Journal of Cell Science & Molecular Biology*, 3(2), 42-45.

Mishra, G., Singh, P., Verma, R., Kumar, S., Srivastav, S., Jha, K.K., Khosa, R.L., 2011. Traditional uses, phytochemistry and pharmacological properties of *Moringa oleifera* plant: An overview. *Der Pharmacia Lettre*, 3(2), 141-164.

Mishra, S., Lakhawat, S., Pandey, H., 2020. *Moringa oleifera*: A Miracle Plant. *Popular Kheti*, 8(2), 29-30.

Moyo, B., Masika, P.J., Muchenje, V., 2012. Antimicrobial activities of *Moringa oleifera* Lam leaf extracts. *African Journal of Biotechnology*, 11(11), 2797-2802.

Muhammad, H.I., Asmawi, M.Z., Khan, N.A.K., 2016. A review on promising phytochemical, nutritional and glycemic control studies on *Moringa oleifera* Lam. in tropical and sub-tropical regions. *Asian Pacific Journal of Tropical Biomedicine*, 6(10), 896-902.

Nair, S., Varalakshmi, K.N., 2011. Anticancer, cytotoxic potential of *Moringa oleifera* extracts on HeLa cell line. *Journal Natural Pharmaceuticals*, 2(3), 138-142.

Nandave, M., Ojha, S.K., Joshi, S., Kumari, S., Arya, D.S., 2009. *Moringa oleifera* leaf extract prevents isoproterenol-induced myocardial damage in rats: evidence for an antioxidant, antiperoxidative, and cardioprotective intervention. *Journal of Medicinal Food*, 12(1), 47-55.

Ndiaye, M., Dieye, A.M., Mariko, F., Tall, A., Faye, B., 2002. Contribution to the study of the anti-inflammatory activity of *Moringa oleifera* (Moringaceae). *Dakar Medical*, 47(2), 210-212.

Obi, A., Egwurugwu, J.N., Ojefa, S.O., Ohamaeme, M.C., Ekweogu, C.N., Ogunnaya, F.U., 2018. Immunomodulatory effects of hydromethanolic extract of *Moringa oleifera* leaf on male wistar rats. *Nigerian Journal of Experimental and Clinical Biosciences*, 6(1), 26-32.

Okwari, O., Dasofunjo, K., Asuk, A., Alagwu, E., Mokwe, C., 2013. Anti-hypercholesterolemic and hepatoprotective effect of aqueous leaf extract of *Moringa oleifera* in rats fed with thermoxidized palm oil diet. *Journal of Pharmaceutical and Biological Sciences*, 8, 57-62.

Ouédraogo, M., Lamien-Sanou, A., Ramdé, N., Ouédraogo, A.S., Ouédraogo, M., Zongo, S.P., Guissou, P.I., 2013. Protective effect of *Moringa oleifera* leaves against gentamicin-induced nephrotoxicity in rabbits. *Experimental and Toxicologic Pathology*, 65(3), 335-339.

Owens, F.S., Dada, O., Cyrus, J.W., Adedoyin, O.O., Adunlin, G., 2020. The effects of *Moringa oleifera* on blood glucose levels: a scoping review of the literature. *Complementary Therapies in Medicine*, 50, 102362.

Oyedepo, T.A., Babarinde, S.O., Ajayeoba, T.A., 2013. Evaluation of anti-hyperlipidemic effect of aqueous leaves extract of *Moringa oleifera* in alloxan induced diabetic rats. *International Journal of Biochemistry Research & Review*, 3(3), 162-170.

Paikra, B.K., Gidwani, B., 2017. Phytochemistry and pharmacology of *Moringa oleifera* Lam. *Journal of Pharmacopuncture*, 20(3), 194-200.

Pan, S.Y., Zhou, S.F., Gao, S.H., Yu, Z.L., Zhang, S.F., Tang, M.K., Ko, K.M., 2013. New perspectives on how to discover drugs from herbal medicines: CAM's outstanding contribution to modern therapeutics. *Evidence-Based Complementary and Alternative Medicine*, 2013, 627375.

Pandey, A., Pradheep, K., Gupta, R., Nayar, E.R., Bhandari, D.C., 2011. 'Drumstick tree'(*Moringa oleifera* Lam.): a multipurpose potential species in India. *Genetic Resources and Crop Evolution*, 58(3), 453-460.

Popoola, J.O., Aworunse, O.S., Oyesola, O.L., Akinnola, O.O., Obembe, O.O., 2020. A systematic review of pharmacological activities and safety of *Moringa oleifera*. *Journal of Herbmed Pharmacology*, 9(3), 174-190.

Popoola, J.O., Obembe, O.O., 2013. Local knowledge, use pattern and geographical distribution of *Moringa oleifera* Lam. (Moringaceae) in Nigeria. *Journal of Ethnopharmacology*, 150(2), 682-691.

Rajanandh, M.G., Kavitha, J., 2010. Quantitative estimation of β-sitosterol, total phenolic and flavonoid compounds in the leaves of *Moringa oleifera*. *International Journal of PharmTech Research*, 2(2), 1409-1414.

Rajbhar, Y.P., Rajbhar, G., Rawat, P.L., Shardulya, S., Kumar, M., 2018. Grow *Moringa* (*Moringa oleifera*), the miracle tree on the earth. *Horticulture International Journal*, 2(4), 166-172.

Randriamboavonjy, J.I., Loirand, G., Vaillant, N., Lauzier, B., Derbré, S., Michalet, S., Tesse, A., 2016. Cardiac protective effects of *Moringa oleifera* seeds in spontaneous hypertensive rats. *American Journal of Hypertension*, 29(7), 873-881.

Saini, R.K., 2013. "Studies on enhancement of carotenoids, folic acid, iron and their bioavailability in *Moringa oleifera* and *in vitro* propagation (Doctoral dissertation, University of Mysore).
Saini, R.K., Sivanesan, I., Keum, Y.S., 2016. Phytochemicals of *Moringa oleifera*: a review of their nutritional, therapeutic and industrial significance. *3 Biotech*, 6(2), 1-14.
Sánchez-Machado, D.I., Lopez-Cervantes, J., Vázquez, N.R., 2006. High-performance liquid chromatography method to measure α-and γ-tocopherol in leaves, flowers and fresh beans from *Moringa oleifera*. *Journal of Chromatography A*, 1105(1-2), 111-114.
Sharifudin, S.A., Fakurazi, S., Hidayat, M.T., Hairuszah, I., Aris Mohd Moklas, M., Arulselvan, P., 2013. Therapeutic potential of *Moringa oleifera* extracts against acetaminophen-induced hepatotoxicity in rats. *Pharmaceutical Biology*, 51(3), 279-288.
Shikwambana, N., Mahlo, S.M., 2020. A survey of antifungal activity of selected south African plant species used for the treatment of skin infections. *Natural Product Communications*, 15(5), 1934578X20923181.
Siddhuraju, P., Becker, K., 2003. Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *Journal of Agricultural and Food Chemistry*, 51(8), 2144-2155.
Singh, A., Navneet, X., 2018. Ethnomedicinal, pharmacological and antimicrobial aspects of *Moringa oleifera* Lam. A review. *The Journal of Phytopharmacology*, 7(1), 45-50.
Singh, A.K., Rana, H.K., Tshabalala, T., Kumar, R., Gupta, A., Ndhlala, A.R., Pandey, A.K., 2020. Phytochemical, nutraceutical and pharmacological attributes of a functional crop *Moringa oleifera* Lam: An overview. *South African Journal of Botany*, 129, 209-220.
Sivasankari, B., Anandharaj, M., Gunasekaran, P., 2014. An ethnobotanical study of indigenous knowledge on medicinal plants used by the village peoples of Thoppampatti, Dindigul district, Tamilnadu, India. *Journal of Ethnopharmacology*, 153(2), 408-423.
Sreelatha, S., Padma, P.R., 2009. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity. *Plant Foods for Human Nutrition*, 64(4), 303-311.
Stevens, G.C., Baiyeri, K.P., Akinnnagbe, O., 2013. Ethno-medicinal and culinary uses of *Moringa oleifera* Lam. in Nigeria. *Journal of Medicinal Plants Research*, 7(13), 799-804.
Sudha, P., Asdaq, S.M., Dhamingi, S.S., Chandrakala, G.K., 2010. Immunomodulatory activity of methanolic leaf extract of *Moringa oleifera* in animals. *Indian Journal of Physiology and Pharmacology*, 54(2), 133-140.
Tejas, G.H., Umang, J.H., Payal, B.N., Tusharbinu, D.R., Pravin, T.R., 2012. A panoramic view on pharmacognostic, pharmacological, nutritional, therapeutic and prophylactic values of *Moringa olifera* Lam. *International Research Journal of Pharmaceuticals*, 3, 1-7.
Thurber, M.D., Fahey, J.W., 2009. Adoption of *Moringa oleifera* to combat under-nutrition viewed through the lens of the "Diffusion of Innovations" theory. *Ecology of Food and Nutrition*, 48(3), 212-225.
Toma, A., Deyno, S., 2014. Phytochemistry and pharmacological activities of *Moringa oleifera*. *International Journal of Pharmacognosy*, 1(4), 222-231.
Vergara-Jimenez, M., Almatrafi, M.M., Fernandez, M.L., 2017. Bioactive components in *Moringa oleifera* leaves protect against chronic disease. *Antioxidants*, 6(4), 91.
Vinoth, B., Manivasagaperumal, R., Balamurugan, S., 2012. Phytochemical analysis and antibacterial activity of *Moringa oleifera* Lam. *International Journal of Research in Biological Science*, 2(3), 98-102.
Waterman, C., Cheng, D.M., Rojas-Silva, P., Poulev, A., Dreifus, J., Lila, M.A., Raskin, I., 2014. Stable, water extractable isothiocyanates from *Moringa oleifera* leaves attenuate inflammation *in vitro*. *Phytochemistry*, 103, 114-122.
Zade, V., Dabhadkar, D., 2015. Antifertility effect of alcoholic extract of *Moringa oleifera* stem bark on estrous cycle and estrogenic activity of female albino rat. *American Journal of Advanced Drug Delivery*, 3(3), 223-235.

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RESEARCH ARTICLE

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Morphology, biological and chemical profiling of three *Polyscias* species, endemic to Mauritius

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ABSTRACT

The aim of the study is to screen the morphological, anatomical, biological, and chemical profiles of the leave extracts of endemic *Polyscias* species namely *P. dichroostachya* (PD), *P. gracilis* (PG), and *P. mauritiana* (PM) from Mauritius. The morphology and anatomy of the leaves were studied using a microscope. Phytochemical screening of extracts using LC-MS/MS was carried out by ionization in both positive and negative modes. The leaves are pinnately compound, hypostomatic, dorsiventral, with a prominent mid vein with secretion cavities, and they can be distinguished by their midrib shape and anatomy. From the molecular mass and fragmentation data, 31 terpenoid saponins, 19 flavonoids, 17 acids, 7 terpenes, and 10 miscellaneous compounds including pyrrolidines and acetylenes in different extracts (hexane, ethanol, and aqueous) were identified. Extracts from the three species using DPPH radical scavenging activity exhibited good to moderate antioxidant activities with IC_{50} values in the range of 2.1-70.8 mg/ml and the ethanolic extract of PD showing the highest activity. Aqueous extracts of PG and PM potently inhibited alpha-glucosidase with IC_{50} values of 0.50 and 2.62 mg/ml. The different leaf extracts exhibited moderate activity against a panel of gram-positive and gram-negative bacteria. This is probably the first report on the extensive chemical profiling and morphology of these endemic *Polysias* spp. from Mauritius and the results indicate that these leaf extracts have beneficial health properties and are thus worth exploiting further.

1. Introduction

The rise in the incidence of diseases together with extensive resistance to existing drugs calls for a continuous quest for new drugs. During the past decades, there has been ongoing drug discovery research, which requires a constantly expanding library of compounds with a wide range of molecular and chemical diversity. Natural products chemistry is considered to be a principal area of research at the interface of chemistry and biology which involves the isolation and characterization of novel bioactive compounds.

Mauritius with an area of 1,865 km^2 is a volcanic island in the Indian Ocean (20°S, 57°E) with a rich botanical diversity consisting of around 700 native species of flowering plants of which 39.5 % are strictly endemic (Baider et al., 2010). This relatively high endemism of Mauritian flora provides a plethora of unique and structurally diverse phytochemicals. However, only a small percentage of the Mauritian endemic plants have been exploited for their medicinal values let alone converted into drug formulation. The Mauritian population has a long tradition of the use of the herbal remedy in the form of "tisanes" or balms to treat diverse ailments such as diabetes, hypertension, gastrointestinal disorders, rheumatism, kidney stones, and anemia, though most of the endemic plants lack phytotherapeutic and scientific evidence to support their medicinal values. There are several scientific reports on the therapeutic potential of endemic plants in Mauritius (Rummun et al., 2018) yet there are only a few studies on the identification of the phytochemicals present in the plants (Świątek et al., 2021). We believe that a substantial fraction of Mauritius native flora resources remain untapped in terms of identification of the metabolites.

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Polyscias, one of the members of the Araliaceae family, consists of approximately 159 species distributed worldwide. There are several scientific reports on the therapeutic potential of various *Polyscias* spp. where several classes of compounds such as terpenes, terpenoids, and terpenoids saponins have been isolated (Ashmawya et al., 2019). Extracts from these *Polyscias* spp. were found to exhibit multiple biological activities such as antibacterial, antifungal, molluscicidal, anti-asthmatic, wound healing, tyrosine kinase inhibitory, immune-stimulant agent (Ashmawya et al., 2020) and hypoglycemic (Luyen et al., 2018). Fifteen species are found in the Mascarene Islands of which six are endemic to Mauritius namely, *P. dichroostachya*, *P. gracilis*, *P. mauritiana*, *P. maraisiana*, *P. neraudiana*, and *P. paniculata*. To the best of our knowledge, the morphoanatomical and phytochemical profiling of these Mauritian *Polyscias* species remains poorly explored. There is one report where four saponins derivatives were identified from the leaves of *P. dichroostachya* (Gopalsamy et al., 1990).

In light of the above, the present study aims at providing morphological, anatomical, biological, and phytochemical profiles of the leaves of the three endemic *Polyscias* species, *P. dichroostachya* (PD), *P. gracilis* (PG), and *P. mauritiana* (PM) of Mauritius.

2. Materials and methods

2.1. Sampling of plant material

Samples of the three endemic *Polyscias* species were collected, identified, preserved, and vouchered at the Mauritius herbarium, Mauritius Sugarcane Industry Research Institute (MSIRI), Réduit. PM (MAU 0005163) leaves were collected from the Black River Gorges National Park (200 26' 14'' S and 570 28' 30'' E), and PD (MAU 0005163) and PG (MAU 0025780) leaves were collected from the MSIRI, Réduit (200 14' 17 ''S and 570 29' 44'' E, and 200 14' 18'' S and 570 29' 45'' E, respectively) (Figure 1), all during the mid-summer season. Fresh mature leaf samples were used for morphological and anatomical studies.

Figure 1. Sites where leaves were collected

2.2. Leaf characterisation

2.2.1. Leaf morphology

The leaf size was measured and the leaf shape was determined using descriptors by Harris and Harris (1994), namely leaf arrangement, venation, size, shape, and attachment.

2.2.2. Leaf Anatomy

2.2.2.1. Stomata

Epidermal impressions of leaves were prepared using clear nail varnish. The film was removed with sticky tape and placed on microscope slides for the determination of stomatal density using an inverted contrasting microscope (Leica DM IL) equipped with an integrated camera, at a magnification of x400. The number of stomata per unit area (mm^{-2}) and length of closed stomata were determined from the images.

2.2.2.2. Transverse sections of leaves

Fresh leaf samples were fixed by placing 1 x 1 cm pieces of leaf tissue excised from the midrib and lamina region in formaldehyde alcohol acetic acid (FAA) (Stasolla and Yeung, 2015) at 4 °C in a refrigerator overnight. The tissues were dehydrated in a graded series of TBA (*t*-butyl alcohol) solutions (six) for 30 minutes each at room temperature. The solutions were made up of 50 ml water, 40 ml 95% ethanol, and 10 ml TBA; 30 ml water, 50 ml 95% ethanol, and 20 ml TBA; 15 ml water, 50 ml 95% ethanol, 35 ml TBA; 45 ml 95% ethanol, 55 ml TBA; 25 ml 100% ethanol, 75 ml TBA; and 100 ml 100% TBA, respectively. Finally, the plant samples were placed in xylene for 30 minutes before being infiltrated in paraffin. Plant sections were cut at an angle of 7 degrees and thickness of 15 µM using a microtome. They were stained with Safranin O (1% w/v) and Fast Green FCF (0.1% w/v) and viewed using an inverted contrasting microscope equipped with a camera (Leica DM IL). The thickness of the midrib was calculated from the images.

2.3. Chemicals and equipment

L-ascorbic acid was purchased from Loba Chemie, India. Diphenyl-2-picrylhydrazyl (DPPH), phosphate buffer, acarbose, and *p*-nitrophenyl-α-D-glucopyranoside (pNPG) were purchased from Sigma Aldrich, England. Broth and Mueller Hinton agar were purchased from Himedia, India. ^{1}H and ^{13}C NMR spectra were recorded at 250 and 62.9 MHz on a Bruker electro-spin NMR spectrometer. DNM-9602 Microplate Reader was used for antioxidant and anti-glucosidase activities.

2.4. Extraction

The air-dried and powdered leaves (100 g) of each species were extracted with *n*-hexane (250 ml) followed by ethanol (250 ml) at room temperature. The respective solvents were concentrated at 50 °C under reduced pressure. For crude aqueous extracts, powdered leaves (20 g) were soaked in boiling water (150 ml) for 24 h, and the filtrate obtained was freeze-dried.

2.4.1. Yields (%)

PM-Hex: 0.8, PM-Et: 2.1, PM-Aq: 0.7
PG-Hex: 0.5, PG-Et: 6.0, PG-Aq: 1.7
PD-Hex: 0.3, PD-Et: 4.0, PD-Aq: 4.2

2.5. Nuclear magnetic resonance and liquid chromatography with tandem mass spectrometry

The ^{1}H and ^{13}C NMR spectra of the different extracts (*n*-hexane, EtOH, aqueous) were recorded at 250 and 62.9 MHz on a Bruker electro spin NMR spectrometer using $CHCl_3$/DMSO/D_2O as solvents.

An HPLC system (Ultimate 3000, Dionex Corporation, Sunnyvale, USA) was integrated with a quadrupole time-of-flight mass analyzer as the detector. A C18 reversed-phase column (XSelect HSS T3 XP, 2.1 mm × 100 mm × 2.5 µM) was used to separate compounds at a flow rate of 0.15 ml/min. The mobile phase consisted of acidified water with 0.1% formic acid (A) and acetonitrile (B). The gradient of the mobile phase was 0-10 min, 10% B; 10-15 min, 10%-90% B; 15-20 min, 90% B; 20-22 min, 90%-10% B; and 22-30 min, 10% B. All samples (1 mg/ml) were reconstituted in 50% aqueous methanol and filtered through a 0.2 µM nylon filter before injection. The sample injection volume was 5 µl. The sample flowed into the mass analyzer and was ionized for detection. The mass ranged from m/z 100-2000 and was set for compound screening using both positive and negative ion modes with two dependent product ion scans using rolling collision energy. Nitrogen gas was used as a nebulizing (40 psi) and curtain (20 psi) gas. The voltage of the ion spray was set at 5500 V and 4500 V for the positive and negative ion modes, respectively. The declustering and the focusing potentials were set at 40 and 300 V, respectively.

2.6. Antibacterial activity

Cultures of gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 10816), and gram-negative bacteria namely *Escherichia coli* (ATCC 22922), *Klebsiella pneumoniae* (ATCC 13883), and *Pseudomonas aeruginosa* (ATCC 27853), were used to evaluate the antibacterial activity of the different extracts using disc diffusion method. The bacteria were streaked on the Mueller Hinton Agar plate using sterile cotton swabs and 10 µl of the sample (400 mg/ml in DMSO) were placed on paper discs (6 mm). Erythromycin was used as the positive control and DMSO as the negative control. All the plates were incubated at 37 °C for 24 h. The activity was determined by measuring the zone of inhibition in millimetres (mm).

2.7. DPPH radical scavenging assay

The *n*-hexane and ethanol extracts were dissolved in DMSO and the water extracts were diluted in water to obtain a solution of 100 mg/ml. DPPH radical scavenging activity was measured at an absorbance of 492 nm (Laulloo et al., 2018). All tests were done in triplicate and ascorbic acid was used as the positive control.

The percentage of radical scavenging inhibition was calculated using the formula:

$$\%\ Inhibition = \frac{\mathrm{A0} - \mathrm{As}}{\mathrm{A0}}\ x\ 100$$

Where A_0 is the absorbance of the blank and A_s is the absorbance of the sample.

2.8. Anti-glucosidase activity

The anti-glucosidase activity assay was carried out on the aqueous extract of all three species. The extracts were diluted to make a stock solution of 5 mg/ml. Two-fold serial dilutions were carried out to obtain concentrations ranging from 1.25 to 0.02 mg/ml. A mixture of 50 µl buffer, 50 µl test sample, and 50 µl α-glucosidase were placed in a 96-well Elisa plate. The mixture was incubated at 37 °C for 5 minutes and 50 µl of pNPG was added followed by incubation for 15 minutes. The absorbance was read at 405 nm (Joondan et al., 2019). The test was carried out in duplicate and acarbose was used as the positive control.

The percentage of anti-glucosidase inhibition was calculated by:

$$\%\ Inhibition = \frac{\mathrm{A0} - \mathrm{As}}{\mathrm{A0}}\ x\ 100$$

Where A_0 is the absorbance of the blank and A_s is the absorbance of the sample.

A_s = *absorbance of sample - absorbance produced by blank*

3. Results and discussion

3.1. Leaf characterization

PD, PG, and PM are perennial eudicot plants. The morphological, and anatomical studies for each species were carried out only on a few leaves collected from one tree as these three endemic plants are endangered.

3.1.1. Morphology

The leaves of the three species are odd-pinnate (Figure 2) and the laminae of leaflets are net-veined and traversed by a prominent midvein. The leaves of PD are composed of nine leaflets while those of PG and PM is composed of seven and five leaflets, respectively. Mature leaflets of PD were around 16-22 cm long and 7-9 cm wide, elliptic, apices cuspidate, leaf margins undulate, and leaf attachment petiolate. Mature leaflets of PG were around 13-15 cm long and 7-10 cm wide, obcordate, leaf margins undulate, and leaf attachment petiolate while PM was around 17-20 cm long and 12-14 cm wide, elliptic with bent cuspidate apices, and sessile.

Leaf morphology is determined by both the genetic makeup and environmental cues (Fritz et al., 2018). Leaves have to adapt to their environment to optimize gas exchange and light capture for photosynthesis. Leaves from PD and PG were collected from the Mauritius Sugarcane Industry Research Institute (MSIRI), Réduit, at an altitude of about 317 meters above sea level, and were therefore subjected to similar environmental conditions with little competition. The PM leaves on the other hand were collected from the damp rainy upland forest of Black River Gorges National Park at an altitude of about 709 meters above sea level and were therefore adapted to another environment.

3.1.2. Anatomy

The leaves of the three *Polyscias* species are hypostomatous with stomata irregularly scattered throughout the abaxial epidermis (Figure 3). This type of stomatal patterning observed is quite common in broad-leaved eudicots. Amphistomaty has been reported to occur mostly in fast-growing herbaceous annuals and slow-growing perennial shrubs and trees (Harrison et al., 2020).

The estimated stomatal density and length of closed stomata for PD, PG, and PM were 151, 85, and 282 stomata per mm^2, and 26.3, 33.3, and 22.9 µm, respectively. PG leaves had the greatest pore size while their stomatal density was considerably lower than the other two species. The PM tree is adapted to a damp and rainy environment and its leaves had the highest stomatal density.

Though the overall shape of stomata is genetically pre-determined, their development is influenced by environmental conditions (Harrison et al., 2020; Driesen et al., 2020). Plants with large and few stomata tend to have higher water-use efficiency as compared with plants that have many but smaller stomata (Drake et al., 2013). It has been reported that small stomatal size can provide a reduction in total leaf pore area and may facilitate faster aperture response than large stomata (Bertolino et al., 2019).

Figure 2. Leaves and leaflets of: A-PD; B-PG; C-PM (Bar = 2 cm)

3.1.3. Transverse sections of leaves

The leaflets of the three species are of the dorsiventral type and have several layers of hypodermal collenchyma on the upper and lower side of the midrib region. Vascular bundles in the midrib are collateral and the xylem tissue is endarch. Several secretory cavities are circularly arranged in the peripheral region of the midrib. The epidermis on both the abaxial and adaxial sides of the lamina are multiseriate and covered with a cuticular layer. Their upper epidermis is four-layered thick while their lower epidermis is two-layered thick. Palisade mesophyll composed of two layers of elongated columnar cells is found below the upper epidermis and spongy mesophyll is situated just above the lower epidermis. Several vascular bundles are centrally located in the leaf lamina.

The leaf anatomy of the three species differs in many ways. In the midrib region of PD leaflets, the vascular tissues are arranged in an arc-shaped belt with two patches just inside the two ends (Figure 4 A). In PG (Figure 4 B) and PM (Figure 4 C), vascular bundles are arranged in a semicircle, though in PM, several vascular bundles are scattered within the semicircle in the central region of their midrib. Furthermore, the spongy mesophyll of PD and PM is made up of loosely arranged cells with intercellular spaces, while that of PG is relatively compact.

The shape of the midrib was different in the three species (Figure 4). The thickness of the midrib and lamina of PD, PG, and PM leaflets were approximately 0.97, 0.96, and 2.1 mm, and 0.44, 0.37, and 0.20 mm, respectively. The thickness of the midrib of the PM leaflets was approximately twice that of the other two *Polycias* species while its lamina was about half their thickness. The diameters of whole veins can reflect their transport capacity and the degree of mechanical support (Sack and Scoffoni, 2013). The thickness of the lamina largely determines the length of the optical path of light through a leaf and is associated with water deficits, low temperature, and high irradiance (Pauli et al., 2017).

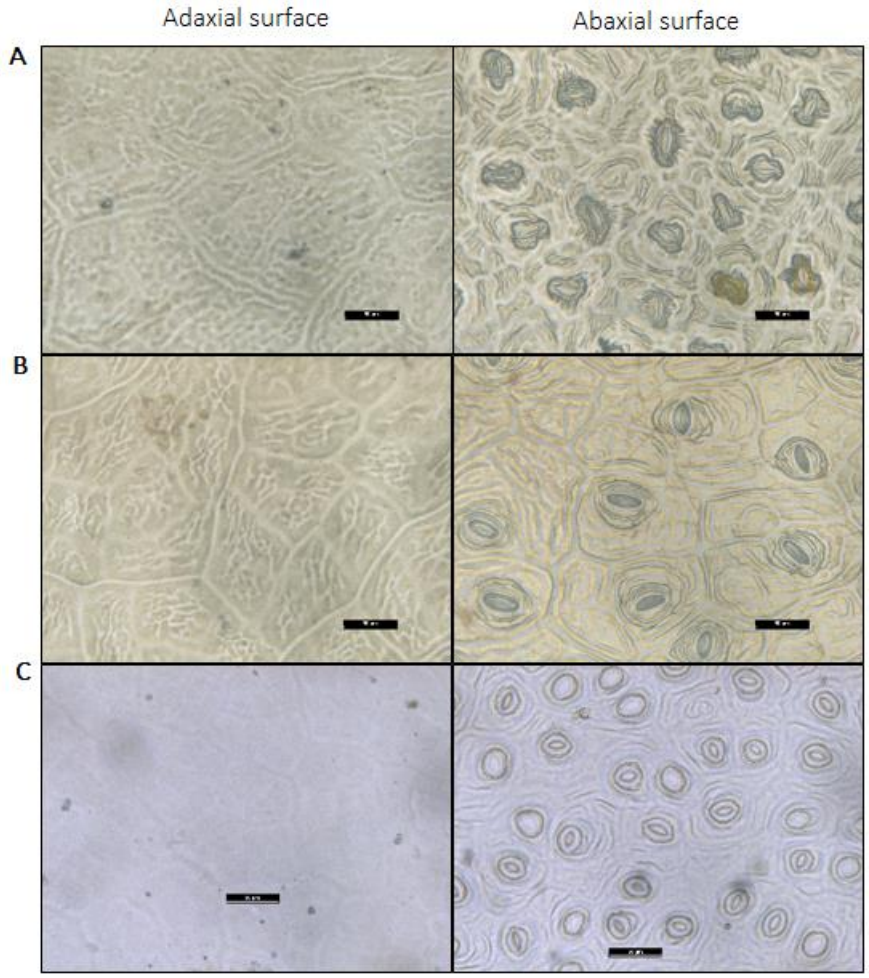


Figure 3. Adaxial surface and abaxial surface with guard cells of leaf blade of: A-PD; B-PG; C-PM (Bar = 50 µm)

3.2. Chemical profiling

The presence of the terpenes and terpenoids were identified in hexane and ethanol extracts of the three species using TLC and test tube testing. Flavonoids in ethanol and aqueous extracts, sterols in aqueous extracts, and saponins were detected in all the extracts. No alkaloids were present in the different extracts.

The NMR spectra of three extracts of air-dried leaves of *Polyscias* spp. showed a difference in chemical signals suggesting the presence of different metabolites in each extract (Figures S1-S6/supplementary file).

In the ^{1}H NMR spectra of the hexane extracts, the major signals were in the region of δ 0.3 to 3.0 and δ 5.1 to 5.6 ppm which were attributed to aliphatic and olefinic protons, respectively. In ^{13}C NMR spectra, the signals in the region of 10 to 60 ppm corresponded to sp^3 hybridized $CH_3/CH_2/CH$ while those in the region of 110 to 120 ppm were due to olefinic carbons. These data indicated mainly the presence of terpenes in the hexane extracts.

In the more polar ethanolic extracts, in addition to the signals due to terpenes moieties, signals in the region δ 3.5 to 5.0 ppm were attributed to sugar moieties. Signals in the region of δ 10 to 55 ppm corresponded to triterpenoid moiety while those in the region of 80 to 110 ppm indicated the presence of sugar moiety. The peaks in the region of 120 to 145 ppm corresponded to sp^2 carbons and the signal at δ 173 ppm were due to the ester group. These data indicated the presence of different terpene/terpenoid saponins and flavonoids in the extracts.

The ^{1}H NMR spectra of the aqueous extracts showed signals at δ 0.8 to 2.7 ppm for aliphatic protons and the more downfield signals (δ 3 to 4 ppm) were attributed to tertiary and allylic methyl groups attached to hydroxyl moiety. These data suggest the presence of steroids in the aqueous extracts.

The different metabolites from the leaf extracts of the PG, PD, and PM were identified using LC-MS/MS. The chemical structure of the phytochemicals was confirmed by the comparison with the literature value and molecular/fragment ions from positive and negative ion modes. It is important to note that this paper does not give the complete structural identification of different species identified in the various extracts.

From the different *Polyscias* species, a total of 31 triterpenoid saponins, 19 flavonoids, 17 derivatives of acids, 7 terpenes, and 10 miscellaneous compounds including pyrrolidines, acetylenes, and sterols were identified. The molecular formula, mass, and fragment ions are given in Table 1.

LC-MS/MS allowed the identification of major saponins and aglycones. The major class of compounds is triterpenoids saponins which consist of a triterpene aglycone with 30 carbon atoms with

one or more sugar moieties (including hexoses, pentoses, methylhexoses). Twenty-one triterpenoid saponins were identified in the negative ion mode while ten were detected in the positive ion mode. Through the relationship of deprotonated ions of triterpenoid saponins, 648-1294 and the protonated *m/z* 620-1220, four aglycones with a molecular weight of 456, 471, 466, and 485 (Figure 5) were detected as daughter ions. The molecular formula of the aglycones was deduced by the loss of sugar molecules from the respective parent ions. Oleanolic acid and hederagenin are the major aglycones while glucose (Glu), glucuronic acid (GluA), glucuronic methyl ester (MeGluA), rhamnose (Rha), methyl pentose (Mepen) and arabinose (Arab) are the main sugar moieties. These sugar molecules were identified by the presence of molecular fragments corresponding to *m/z* 163, 177, 191, 147, 146, and 133.

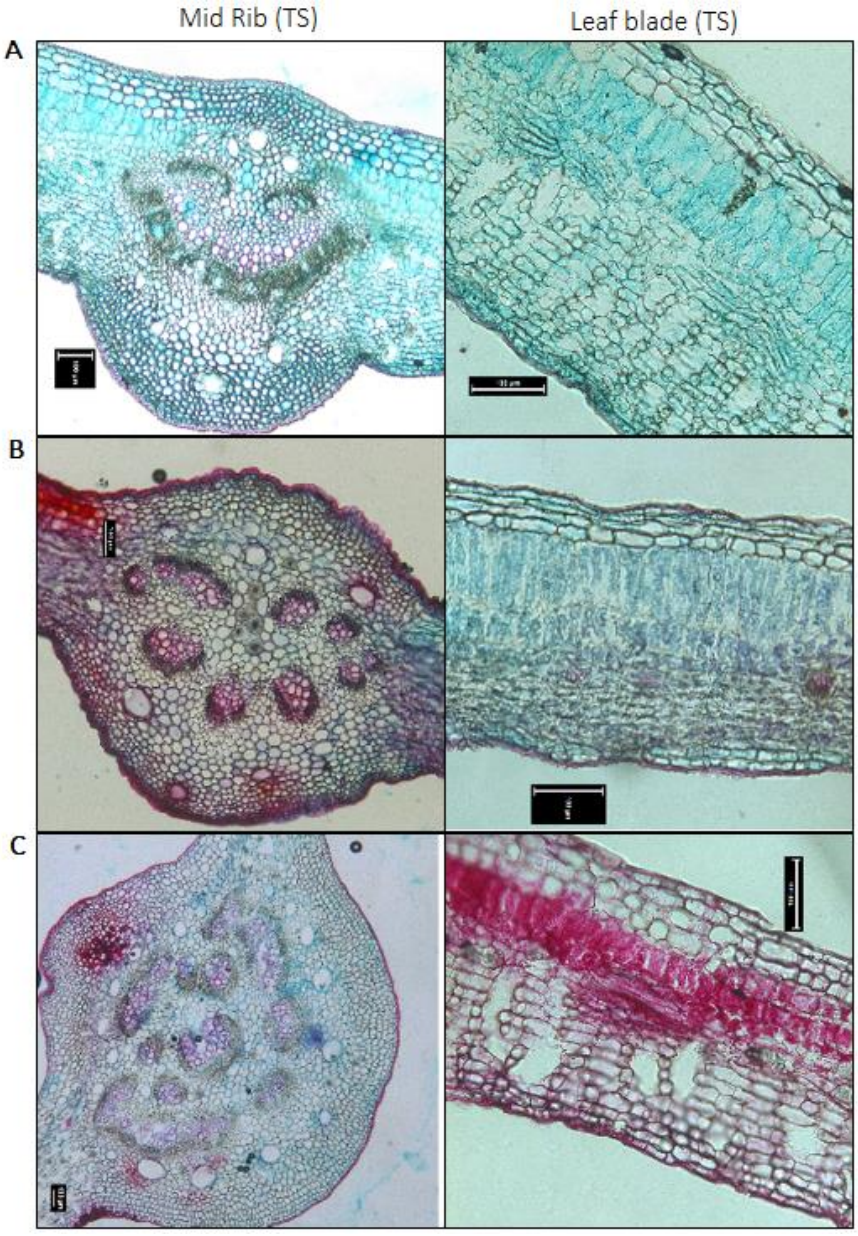


Figure 4. Leaf transverse sections showing the midrib and leaf blade of: A-PD; B-PG; C-PM (Bar = 100 µm)

Flavonoids are fifteen-carbon skeletons consisting of two benzene rings linked via a heterocyclic pyrene ring and may contain glycosides. The main flavonoid aglycones are kaempferol and quercetin containing one and two sugar moieties. Two flavones, namely acacetin, and vitexin (F1, F7) and catechin (F3) and its methoxy derivative (F5) were identified using fragmentation patterns in mass spectra. The two main flavonols kaempferol (F2), quercetin (F4), and their methoxy derivatives (tamarixetin, F6) containing one to four sugar moieties were detected (Figure 6) (Table 1).

The presence of the fragment ion *m/z* 191 in the mass spectra of the six acids (A8, A10, A11, A13, A15, A16) showed them to be quinic acid derivatives. A9, A12, and A14 were identified as acid glycosides based on the loss of sugar moieties from the parent ion (Table 2). Two pyrrolidine derivatives containing carboxylic acid and hydroxyl ethyl residue were detected. Steroid derivatives namely ergosterol, campestanol, and sitosterol were also identified (Table 2).

3.3. Biological properties

3.3.1. Antibacterial activity

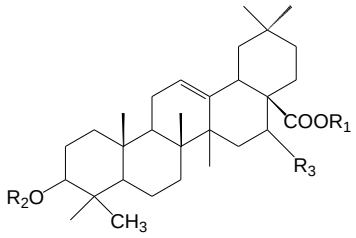
Plant constituents such as flavonoids, terpenes, alkaloids, and tannins are known to display antibacterial activity (Tankeo et al., 2015). The different leaf extracts exhibited moderate inhibition (8-10 mm) at the concentration of 400 mg/ml against a set of gram-positive and gram-negative bacteria. The hexane extracts of the different species showed the highest inhibitory activity which may be attributed to the presence of several terpenes. The ethanolic extract containing several saponins was found to exhibit moderate activity in line with Maritim (Winnie et al., 2019).

Table 1. Triterpenoids and flavanoids detected from the leaves extract of *Polyscias* spp.

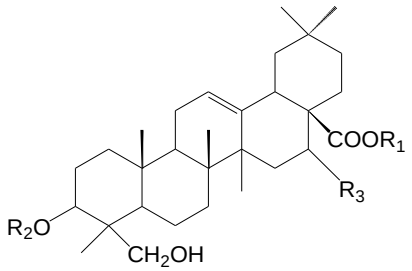
Compound	Formula	m/z	$[M-H]^-$ m/z	$[M+H]^+$ m/z	MS/MS	PG			PD			PM			Reference
						Hex	EtOH	Aq	Hex	EtOH	Aq	Hex	EtOH	Aq	
TS1	$C_{30}H_{48}O_4$	472		473	463, 350, 301, 293		+		+	+	+				(Mitaine-Offer et al., 2004)
TS2	$C_{32}H_{49}O_3$	480		481	467, 425, 409, 305, 191			+							(Masruri et al., 2007)
TS3	$C_{35}H_{56}O_9$	620		621	620, 474, 424, 402		+								(Eaton et al., 2015)
TS4	$C_{37}H_{56}O_8$	628		629	509,482,467,425,403	+	+								(Masruri et al., 2007)
TS5	$C_{36}H_{58}O_{10}$	648	647		603, 471			+		+			+		*
TS6	$C_{37}H_{58}O_{10}$	662	661		570, 602,615			+							*
TS7	$C_{41}H_{66}O_{12}$	750	749	751	471, 585, 603, 749			+					+		(Winnie et al., 2019)
TS8	$C_{41}H_{66}O_{13}$	766		767	739, 585, 455, 437								+		(Ashmawya et al., 2020)
TS9	$C_{42}H_{68}O_{13}$	780	779		455, 732	+	+						+		(Masruri et al., 2007)
TS10	$C_{41}H_{65}O_{14}$	781		782	471, 453					+					(Huan et al., 1998)
TS11	$C_{42}H_{66}O_{14}$	794	793		473, 585, 602, 747	+	+			+	+	+	+		(Paphassarang et al., 1989)
TS12	$C_{42}H_{68}O_{14}$	796	795		601, 471	+						+		+	*
TS13	$C_{43}H_{68}O_{14}$	808	807		495, 591			+							(Sugimoto et al., 2017; Elgindi et al., 2015)
TS14	$C_{42}H_{68}O_{15}$	810	809		485, 617, 765		+			+					(Nascimento et al., 2019)
TS15	$C_{42}H_{68}O_{15}$	810		811	765, 603, 471		+		+	+					*
TS16	$C_{44}H_{72}O_{14}$	824	823		577, 603, 739, 777		+	+					+		(Elgindi et al., 2015)
TS17	$C_{44}H_{72}O_{14}$	824	823		455			+							*
TS18	$C_{44}H_{69}O_{15}$	837	836		225, 340, 452, 564, 772, 790	+			+			+			*
TS19	$C_{43}H_{66}O_{16}$	838	839		471			+							*
TS20	$C_{48}H_{77}O_{17}$	925		924	762, 601, 583, 565, 471, 455, 437, 309								+		(Nascimento et al., 2019)
TS21	$C_{48}H_{78}O_{18}$	942		943	455, 437, 408, 309								+		(Paphassarang et al., 1990)
TS22	$C_{49}H_{78}O_{19}$	970	969		247, 441, 453			+					+		(Do et al., 2020)
TS23	$C_{48}H_{76}O_{20}$	972	973		603, 323								+		(Elgindi et al., 2015)
TS24	$C_{48}H_{75}O_{21}$	986		987	453, 471		+		+					+	*
TS25	$C_{48}H_{74}O_{21}$	986	985		457, 469			+					+		*
TS26	$C_{54}H_{88}O_{24}$	1120	1119		471, 585, 603, 749			+		+			+		(Hanh et al., 2016)
TS27	$C_{59}H_{96}O_{26}$	1220		1221	455, 437, 409, 293		+	+					+		(Njateng et al., 2017)
TS28	$C_{60}H_{98}O_{27}$	1250	1249		255, 731		+								(Huan et al., 1998)
TS29	$C_{60}H_{98}O_{28}$	1266	1265		205, 247, 469, 749	+	+	+					+		(Huan et al., 1998)
TS30	$C_{60}H_{97}O_{29}$	1282	1281		603, 465, 362					+					(Hanh et al., 2016)
TS31	$C_{60}H_{94}O_{30}$	1294	1293		258, 453, 746, 777		+								*
F1	$C_{16}H_{12}O_5$	284		285	240									+	(Lin et al., 2019)
F2	$C_{15}H_{10}O_6$	286		287	270, 226, 106	+	+	+	+		+				*
F3	$C_{15}H_{14}O_6$	290	289		146, 174		+						+		(Li and Seeram, 2018)
F4	$C_{15}H_{10}O_7$	302		303	282, 109		+	+	+	+	+				(Figat et al., 2020)
F5	$C_{16}H_{16}O_6$	304	303		266, 248, 175, 147, 134				+	+					*
F6	$C_{16}H_{12}O_7$	316		317	298, 254, 106	+	+	+			+		+		(Sugimoto et al., 2017)

Compound	Formula	m/z	$[M-H]^-$ m/z	$[M+H]^+$ m/z	MS/MS	PG			PD			PM			Reference
						Hex	EtOH	Aq	Hex	EtOH	Aq	Hex	EtOH	Aq	
F7	$C_{21}H_{20}O_{10}$	432	431		153, 205			+		+			+		(Li and Seeram, 2018)
F8	$C_{21}H_{20}O_{11}$	448	447		161, 269, 398		+	+		+			+	+	(Eaton et al., 2015; Elgindi et al., 2015; Lin et al., 2019)
F9	$C_{21}H_{20}O_{12}$	464	463	465	254, 271, 300			+					+		(Bedir et al., 2001)
F10	$C_{21}H_{18}O_{13}$	478	477		151, 179, 301		+	+					+	+	*
F11	$C_{27}H_{30}O_{15}$	594	593		207	+									(Ashmawya et al., 2019)
F12	$C_{28}H_{32}O_{15}$	608		609	591, 577, 559, 531, 515, 475				+	+					(Sugimoto et al., 2017)
F13	$C_{27}H_{30}O_{16}$	611	610		564, 543, 450				+	+					(Figat et al., 2020)
F14	$C_{28}H_{32}O_{16}$	624	625		607, 565, 538, 521, 465, 477				+	+	+				*
F15	$C_{27}H_{30}O_{17}$	626	627		607, 565, 538,521, 465										*
F16	$C_{23}H_{40}O_{20}$	636		637	619, 587, 559, 476	+		+							*
F17	$C_{30}H_{32}O_{19}$	696		697	311						+				*
F18	$C_{33}H_{40}O_{20}$	757		758	283					+					*
F19	$C_{39}H_{52}O_{25}$	920		921	627, 609, 591, 331		+		+						*

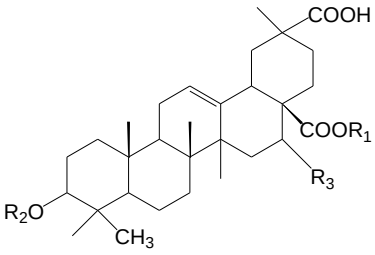
*Based on mass fragments



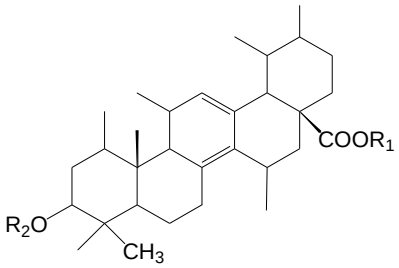
TS8: R_1= H, R_2= Arab-Glu, R_3=OH
TS9: R_1= H, R_2= Glu-Glu, R_3=H
TS10: R_1= H, R_2= GluA-Arab, R_3=OH
TS11: R_1= H, R_2= GluA-Rham, R_3=OH
TS13: R_1= Glu, R_2= MeGluA, R_3=H`
TS17: R_1= H, R_2= MegluA -Glu, R_3=OH
TS18: R1= H, R2= MegluA - MegluA, R_3=H
TS20: R_1= Ara, R_2= MegluA-Arab, R_3=OH
TS21: R_1= Glu, R_2= Glu-Glu, R_3=H
TS22: R_1= Glu, R_2= MeGluA-Glu, R_3=H
TS24: R_1= GluA, R_2= GluA-Glu, R_3=OH
TS26: R_1= Glu, R_2= Glu-Glu-Glu, R_3=OH
TS27: R_1= Rham-Glu-Glu, R_2= Rha-Ara, R_3=OH
TS28: R_1= Glu-Rha, R_2= Glu-Glu-Glu, R_3=H
TS30: R_1= Glu-Glu, R_2= GluA(Glu)-Glu, R_3=H
TS31: R_1= GluA-Glu, R_2= GluA(Glu)-Glu, R_3=H



TS1: R_1= H, R_2=H, R_3= H
TS3: R_1= H, R_2=Ara, R_3= OH
TS5: R_1= H, R_2=GluA, R_3= H
TS6: R_1= H, R_2=MeGluA, R_3= H
TS7: R_1= H, R_2=Arab-Rham, R_3= H
TS12: R_1= H, R_2=Glu-Glu, R_3= H
TS15: R_1= H, R_2= Arab-MeGluA, R_3=OH
TS16: R_1= H, R_2= MeGluA-Glu, R_3=H
TS19: R_1= H, R_2= MeGluA-GluA, R_3=H
TS29: R_1= Glu-Rha, R_2= Glu-Glu-Glu, R_3=H

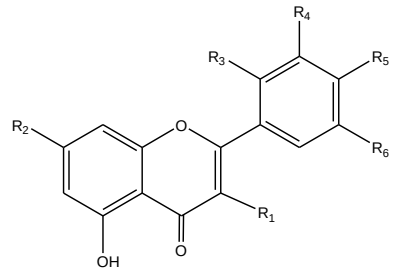


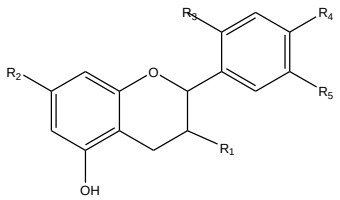
TS14: R_1= Glu, R_2=Glu
TS23: R_1= H, R_2= MegluA-Glu-Ara
TS25: R_1= Glu, R_2= GluA-Glu



TS2: R_1= CH3, R_2=H
TS4: R_1= Glu, R_2=H

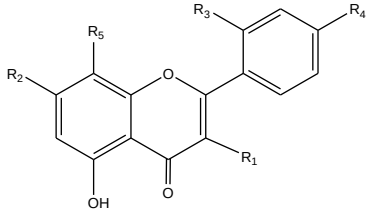
Figure 5. Proposed structures of triterpenoid saponins





F2: R_1= OH; R_2= OH; R_3=H, R_4=H; R_5=OH; R_6=H
F4: R_1= OH; R_2= OH; R_3=H, R_4=OH; R_5=OH, R_6=H
F6: R_1= OH; R_2= OH; R_3=H, R_4=OH; R_5=OCH_3, R_6=H
F8: R_1= ORham; R_2= OH; R_3=H, R_4=OH; R_5=OH, R_6=H
F9: R_1= OGlu; R_2= OH; R_3=H, R_4=H; R_5=OH, R_6=OH
F10: R_1= OGluA; R_2= OH; R_3=H, R_4=OH; R_5=OH, R_6=H
F11: R_1= ORham; R_2= ORham; R_3=H, R_4=H; R_5=OH, R_6=OH
F12: R_1= ORham; R_2= ORham; R_3=H, R_4=H; R_5=OCH_3, R_6=OH
F13: R_1= OGlu-Rham; R_2= OH; R_3=OH, R_4=H; R_5=OH, R_6=OH
F14: R_1= OGlu; R_2= ORham; R_3=H, R_4=H; R_5=OCH_3, R_6=OH
F15: R_1= OGlu; R_2= OGlu; R_3=OH, R_4=H; R_5=OH, R_6=H
F16:R_1= ORham; R_2= OMegluA; R_3=H, R_4=H; R_5=OCH_3, R_6=H
F17: R_1= OMegluA; R_2= OMegluA; R_3=H, R_4=H; R_5=OCH_3, R_6=OH
F18: R_1= OGlu-Rham; R_2= ORham; R_3=OH, R_4=H; R_5=OH, R_6=H
F19: R_1= OGlu-Rham; R_2= OGlu-Rham; R_3=OH, R_4=H; R_5=OH, R_6=H

F3:R_1=OH; R_2= OH; R_3=H, R_4=OH, R_5=OH
F5:R_1=OH; R_2= OCH_3; R_3=H, R_4=OH, R_5=OH



F1:R_1= H; R_2= OH; R_3=H, R_4=OCH_3, R_5=H
F7: R_1= H; R_2=OH; R_3=H, R_4=OH; R_5=Glu

Figure 6. Proposed structure of flavanoids

Table 2. Proposed structure of acids, terpenes and miscellaneous compounds detected in the extracts of *Polyscias* spp.

Identified compounds		m/z	$[M-H]^-$	$[M+H]^+$	MS	PG			PD			PM			Reference
Name/Formula			m/z	m/z		Hex	EtOH	Aq	Hex	EtOH	Aq	Hex	EtOH	Aq	
Acids															
A1	Malic Acid ($C_4H_6O_5$)	134	133		115	+	+	+			+	+		+	(Figat et al., 2020)
A2	Procatechuic acid ($C_7H_6O_4$)	154	153		109	+	+	+						+	(Lin et al., 2019)
A3	3-(4-Hydroxyphenyl) propionic acid ($C_9H_{10}O_3$)	166	165		121	+						+			(Ashmawy a et al., 2020)
A4	Caffeic acid ($C_9H_8O_4$)	180	179		115, 132, 163	+									(Lin et al., 2019)
A5	Quinic acid ($C_7H_{12}O_6$)	192	191		111, 129		+	+			+			+	(Willems et al., 2016)
A6	Methyl 2,4-dihydroxy-3,6-dimethylbenzoate (Atraric acid) ($C_{10}H_{12}O_4$)	196	195	197	160, 135, 114				+	+				+	(Willems et al., 2016)
A7	Hydroxy-octadecatrienoic acid ($C_{18}H_{30}O_3$)	294	293		236, 220, 221, 218, 194	+	+	+		+		+	+		(Figat et al., 2020)
A8	5-*O*-Caeffoylquinic acid ($C_{16}H_{18}O_8$)	338	337		172, 191		+	+					+		(Willems et al., 2016)
A9	Caeffeoyl glucose ($C_{15}H_{18}O_9$)	342	341	343	135, 161, 179, 221, 251	+	+	+		+	+		+		(Said et al., 2017)
A10	5-*O*-Caffeoylquinic acid (chlorogenic acid) ($C_{16}H_{18}O_9$)	354	353		179, 191	+	+	+					+	+	*
A11	5-*O*-Feruloyquinic acid ($C_{17}H_{20}O_9$)	368	367		191	+	+	+					+	+	*
A12	Caffeoyl-glucaric acid ($C_{15}H_{16}O_{11}$)	372	371		191, 209	+									*
A13	Quinic acid derivative	407	406		282, 190, 161, 135						+				*
A14	Procatechuic acid containing glucose and gluconic ($C_{25}H_{32}O_{10}$)	492		490/491/494	467, 425, 407, 177, 163			+	+	+					*
A15	3,5-di-*O*-caffeoyl-quinic acid ($C_{25}H_{24}O_{12}$)	516	515		179, 191	+	+	+		+			+	+	(Figat et al., 2020)
A16	Quinic acid derivative	548	547		134, 149, 191			+							*

Identified compounds		m/z	$[M-H]^-$	$[M+H]^+$	MS	PG			PD			PM			Reference
Name/Formula			m/z	m/z		Hex	EtOH	Aq	Hex	EtOH	Aq	Hex	EtOH	Aq	
A17	Phenolic acid derivative	584	583		195, 534		+								(Sliwinska et al., 2018)
Terpenes															
T1	Terpene ($C_{15}H_{18}$)	198		199	178, 159, 156, 148, 137	+									(Ashmawy a et al., 2018)
T2	Sesquiterpene ($C_{15}H_{24}$)	204		203	147, 121, 119, 109, 107				+	+					(Ashmawy a et al., 2018)
T3	Phytol ($C_20H_{40}O$)	296	295	297	185, 277	+	+			+					(Ashmawy a et al., 2018)
T4	Diterpenoid ($C_{20}H_{34}O_3$)	322		323				+							(Ashmawy a et al., 2020)
T5	Diterpenoid ($C_{20}H_{26}O_4$)	330		329	284, 106		+								(Tang et al., 2011)
T6	Diterpenoid ($C_{21}H_{30}O_4$)	346		347	240, 283, 106	+	+	+		+	+				*
T7	Squalene ($C_{30}H_{50}$)	410		411	239, 133		+								(Bertilino et al., 2019)
Others															
	5-Oxopyrrolidine-2-carboxylic acid ($C_5H_7NO_3$)	129		130	112		+								(Sugimoto et al., 2017)
	2-Hydroxyethyl 5-oxopyrrolidine-2-carboxylate ($C_7H_{11}NO_4$)	173		174	165, 163, 162, 153				+						(Sugimoto et al., 2017)
	Falcarinol ($C_{17}H_{24}O$)	244		243	241, 232, 223, 214, 133	+		+							(Ashmawy a et al., 2020)
	Heptadeca-1,8-diene-4,6-diyne-3-ol-10-one ($C_{17}H_{22}O_2$)	258		259				+	+			+			(Ashmawy a et al., 2020)
	Pinoresinol ($C_{20}H_{22}O_6$)	358		359	340, 312/ 350, 293, 237	+	+	+	+	+	+				(Njateng et al., 2017)
	Ergosterol ($C_{28}H_{44}O$)	396		397	379, 239, 229, 177, 133			+		+					(Münger et al., 2018)
	Campestanol ($C_{28}H_{50}O$)	402	401		121, 181, 223,312, 357		+	+							(Münger et al., 2018)
	Canthoside A ($C_{18}H_{26}O_{12}$)	434	433		227, 387		+								(Lin et al., 2019)
	β-sitosterol ($C_{29}H_{50}O$)	414	415		406, 350,307,293	+	+		+	+	+				(Ragasa et al., 2015)

*Based on mass fragments

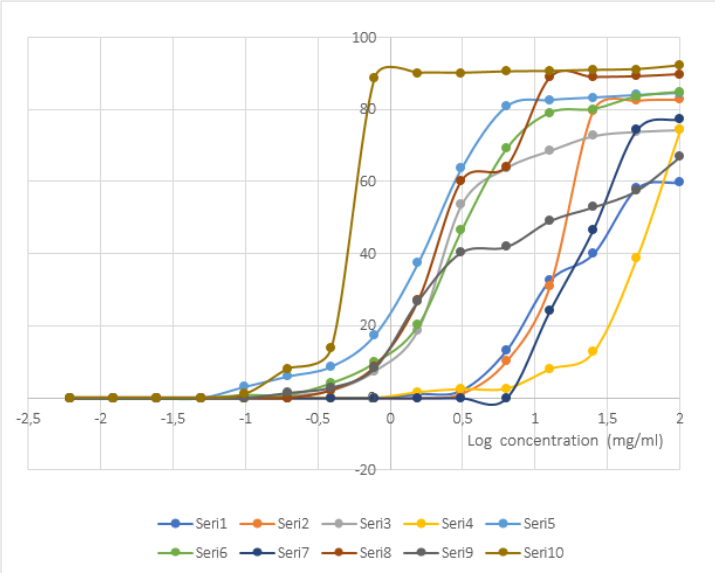


Figure 7. Percentage inhibition of different extracts of *Polyscias* spp.

Table 3. Radical scavenging activity SC_{50} (mg/ml) of the different extracts

Extracts	PM	Category	PG	Category	PD	Category
Hex	14.6	Weak	48.8	Weak	46.1	Weak
EtOH	2.3	Strong	7.4	Moderate	2.2	Strong
Aq	16.8	Moderate	4.6	Strong	3.2	Strong

Ascorbic acid SC_{50}: 0.6 mg/ml

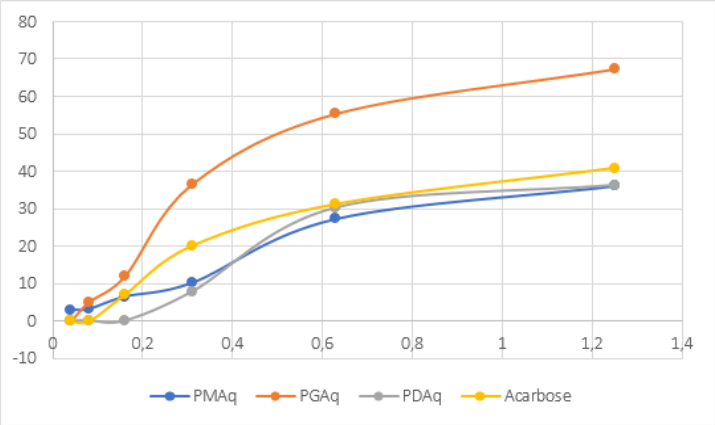


Figure 8. Anti-α-glucosidase activity of aqueous extract of *Polyscias* spp.

3.3.2. Antioxidant capacity

Extracts from the three species showed significant levels of radical scavenging activity and were dose-dependent (Figure 7) with IC_{50} values in the range of 2.1-70.8 mg/ml (Table 3). The ethanolic and aqueous extracts were found to be potent DPPH scavengers as compared to the hexane extracts. The presence of several flavonoids (including acacetin, kaempferol, catechin, and quercetin), glucosides derivatives, and quinic acid derivatives (Figure 5 and Table 1) contributes to their anti-oxidant activity (Njateng et al., 2017).

3.3.3. Anti-α-glucosidase activity

The inhibitory effects of the aqueous extracts were evaluated against yeast α-glucosidase with acarbose as the positive control. Pentacyclic triterpenoids saponins have been reported to exhibit α-amylase and α-glucosidase activity (Luyen et al., 2018). The presence of glucuronic acid and glucosidic moieties on the aglycones were essential components for α-glucosidase inhibition (Hanh et al., 2016). The extracts of PG and PM potently inhibited α-glucosidase with IC_{50} values of 0.50 and 2.62 mg/ml and were comparable to the acarbose with an IC_{50} value of 2.40 mg/ml (Figure 8).

4. Conclusions

The leaves of the three endemic *Polyscias* species can be discriminated based on the shape and arrangement of the vascular tissue of the midrib. The extracts of these leaves showed to exhibit promising antioxidant and anti-glucosidase activities. From the phytochemical analysis, it was found that the major components were triterpenoid saponins, flavonoids, and acids. It is possible that the presence of these metabolites could be responsible for the observed biological properties.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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References

Ashmawya, N.S., Gad, H.A., Al-Musayeib, N., El-Ahmady, S.H., Ashour, M.L., Singab, A.N. B., 2019. Phytoconstituents from *Polyscias guilfoylei* leaves with histamine-release inhibition activity. *Zeitschrift für Naturforschung C*, 74(5-6), 145-150.

Ashmawya, N.S., Gad, H.A., Ashour, M.L., El-Ahmady, S.H., Singab, A.N.B., 2020. The genus *Polyscias* (Araliaceae): A phytochemical and biological review. *Journal of Herbal Medicine*, 23, 100377.

Ashmawya, N.S., Gad, H.A., Ashoura, M.L., El-Ahmadya, S.H., Singab, A.N.B., 2018. Comparative study on the volatile constituents of *Polyscias guilfoylei* and *Polyscias balfouriana* leaves. *Medicinal & Aromatic Plants*, 7(6), 321.

Baider, C., Florens, F.V., Baret, S., Beaver, K., Matatiken, D., Strasberg, D., Kueffer, C., 2010. Status of plant conservation in oceanic islands of the Western Indian Ocean. In Proceedings of the 4th global botanic gardens congress (Vol. 2, pp. 1-7). Dublin: National Botanic Gardens of Ireland.

Bedir, E., Toyang, N.J., Khan, I.A., Walker, L.A., Clark, A.M., 2001. A new dammarane-type triterpene glycoside from *Polyscias fulva*. *Journal of Natural Products*, 64(1), 95-97.

Bertolino, L.T., Caine, R.S., Gray, J.E., 2019. Impact of stomatal density and morphology on water-use efficiency in a changing world. *Frontiers in Plant Science*, 10, 225.

Do, V.M., Tran, C.L., Nguyen, T.P., 2020. Polysciosides J and K, two new oleanane-type triterpenoid saponins from the leaves of *Polyscias fruticosa* (L.) harms. cultivating in An Giang Province, Viet Nam. *Natural Product Research*, 34(9), 1250-1255.

Drake, P.L., Froend, R.H., Franks, P.J., 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany*, 64(2), 495-505.

Driesen, E., Van den Ende, W., De Proft, M., Saeys, W., 2020. Influence of environmental factors light, CO_2, temperature, and relative humidity on stomatal opening and development: A review. *Agronomy*, 10(12), 1975-2002.

Eaton, A.L., Brodie, P.J., Callmander, M.W., Rakotondrajaona, R., Rakotobe, E., Rasamison, V.E., Kingston, D.G., 2015. Bioactive Oleanane Glycosides from *Polyscias duplicata* from the Madagascar Dry Forest. *Natural Product Communications*, 10(4), 567-570.

Elgindi, M.R., Melek, F.R., Hassan, M.A., Abdelaziz, H.S., 2015. Saponins isolated from *Polyscias guilfoylei* F. Araliaceae. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 6(3), 545–549.

Figat, R., Śliwińska, A., Stochmal, A., Soluch, A., Sobczak, M., Zgadzaj, A., Pietrosiuk, A., 2020. Antigenotoxic, anti-photogenotoxic, and antioxidant properties of *Polyscias filicifolia* shoots cultivated *in vitro*. *Molecules*, 25(5), 1090.

Fritz, M.A., Rosa, S., Sicard, A., 2018. Mechanisms underlying the environmentally induced plasticity of leaf morphology. *Frontiers in Genetics*, 9, 478.

Gopalsamy, N., Gueho, J., Julien, H.R., Owadally, A.W., Hostettmann, K., 1990. Molluscicidal saponins of *Polyscias dichroostachya*. *Phytochemistry*, 29(3), 793-795.

Hanh, T.T.H., Dang, N.H., Dat, N.T., 2016. α-Amylase and α-glucosidase inhibitory saponins from *Polyscias fruticosa* leaves. *Journal of Chemistry*, 2016, 2082946.

Harris, J.G., Harris, M.W., 1994. Plant identification terminology: an illustrated glossary (No. QK9 H37 2001). Utah: Spring Lake Publishing.

Harrison, E.L., Arce Cubas, L., Gray, J.E., Hepworth, C., 2020. The influence of stomatal morphology and distribution on photosynthetic gas exchange. *The Plant Journal*, 101(4), 768-779.

Huan, V.D., Yamamura, S., Ohtani, K., Kasai, R., Yamasaki, K., Nham, N.T., Chau, H.M., 1998. Oleanane saponins from *Polyscias fruticosa*. *Phytochemistry*, 47(3), 451-457.

Joondan, N., Laulloo, S.J., Caumul, P., Kharkar, P.S., 2019. Antioxidant, antidiabetic and anticancer activities of L-Phenylalanine and L-Tyrosine ester surfactants: *in vitro* and *in silico* studies of their interactions with macromolecules as plausible mode of action for their biological properties. *Current Bioactive Compounds*, 15(6), 610-622.

Laulloo, S.J., Bhowon, M.G., Soyfoo, S., Chua, L.S., 2018. Nutritional and biological evaluation of leaves of *Mangifera indica* from Mauritius. *Journal of Chemistry*, 2018, 1-9.

Li, C., Seeram, N.P., 2018. Ultra-fast liquid chromatography coupled with electrospray ionization time-of-flight mass spectrometry for the rapid phenolic profiling of red maple (*Acer rubrum*) leaves. *Journal of Separation Science*, 41(11), 2331-2346.

Lin, M., Han, P., Li, Y., Wang, W., Lai, D., Zhou, L., 2019. Quinoa secondary metabolites and their biological activities or functions. *Molecules*, 24(13), 2512.

Luyen, N.T., Dang, N.H., Binh, P.T.X., Hai, N.T., Dat, N.T., 2018. Hypoglycemic property of triterpenoid saponin PFS isolated from *Polyscias fruticosa* leaves. *Anais da Academia Brasileira de Ciências*, 90, 2881-2886.

Masruri, E., Elvina, D.I., Kristianingsih, E.P.U., Rahman, M.F., Rurini, R., 2007. Indentification of triterpenoid compound from *Polyscias fruticosa* Harms (Araliaceae) root bark. *In International Conference on Chemical Sciences*, Yogyakarta- Indonesia, 24-26 May.

Mitaine-Offer, A.C., Tapondjou, L.A., Lontsi, D., Sondengam, B.L., Choudhary, M.I., Lacaille-Dubois, M.A., 2004. Constituents isolated from *Polyscias fulva*. *Biochemical Systematics and Ecology*, 6(32), 607-610.

Münger, L.H., Boulos, S., Nyström, L., 2018. UPLC-MS/MS based identification of dietary steryl glucosides by investigation of corresponding free sterols. *Frontiers in Chemistry*, 6, 342.

Nascimento, Y.M., Abreu, L.S., Lima, R.L., Costa, V.C.O., Melo, J.I.M.D., Braz-Filho, R., Tavares, J.F., 2019. Rapid characterization of triterpene saponins from *Zornia brasiliensis* by HPLC-ESI-MS/MS. *Molecules*, 24(14), 2519.

Njateng, G.S.S., Du, Z., Gatsing, D., Mouokeu, R.S., Liu, Y., Zang, H.X., Kuiate, J.R., 2017. Antibacterial and antioxidant properties of crude extract, fractions and compounds from the stem bark of *Polyscias fulva* Hiern (Araliaceae). *BMC Complementary and Alternative Medicine*, 17(1), 99.

Paphassarang, S., Raynaud, J., Lussignol, M., Becchi, M., 1989. Triterpenic glycosides from *Polyscias scutellaria*. Phytochemistry, 28(5), 1539-1541.

Paphassarang, S., Raynaud, J., Lussignol, M., Cabalion, P., 1990. A new oleanolic glycoside from *Polyscias scutellaria*. *Journal of Natural Products*, 53(1), 163-166.

Pauli, D., White, J.W., Andrade-Sanchez, P., Conley, M.M., Heun, J., Thorp, K.R., Gore, M.A., 2017. Investigation of the influence of leaf thickness on canopy reflectance and physiological traits in upland and Pima cotton populations. *Frontiers in Plant Science*, 8, 1405.

Ragasa, C.Y., Ebajo, V.D., De Los Reyes, M.M., Brkljaca, R., Urban, S., 2015. Chemical constituents of *Polyscias nodosa*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 6(5), 1210-1214.

Rummun, N., Neergheen-Bhujun, V.S., Pynee, K.B., Baider, C., Bahorun, T., 2018. The role of endemic plants in Mauritian traditional medicine–Potential therapeutic benefits or placebo effect?. *Journal of Ethnopharmacology*, 213, 111-117.

Sack, L., Scoffoni, C., 2013. Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. *New Phytologist*, 198(4), 983-1000.

Said, B.R., Arafa, I.H., Usam A,M., Abdullah Sulaiman, A.A., Kowalczyk, M., Moldoch, J., Stochmal, A., 2017. Tentative characterization of polyphenolic compounds in the male flowers of *Phoenix dactylifera* by liquid chromatography coupled with mass spectrometry and DFT. *International Journal of Molecular Sciences*, 18(3), 512.

Sliwinska, A.A., Syklowska-Baranek, K., Kosmider, A., Granica, S., Miszczak, K., Nowicka, G., Pietrosiuk, A., 2018. Stimulation of phenolic compounds production in the *in vitro* cultivated *Polyscias filicifolia* Bailey shoots and evaluation of the antioxidant and cytotoxic potential of plant extracts. *Acta Societatis Botanicorum Poloniae*, 87(2), 1-16.

Stasolla, C., Yeung, E.C., 2015. 'Paraffin and Polyester Waxes' in Edward Chee Tak Yeung, Claudio Stasolla, Michael John Sumner and Bing Quan Huang, eds. Plant Microtechniques and Protocols. Cham Springer International Publishing, pp. 45-66.

Sugimoto, S., Yamano, Y., Khalil, H.E., Otsuka, H., Kamel, M.S., Matsunami, K., 2017. Chemical structures of constituents from the leaves of *Polyscias balfouriana*. *Journal of Natural Medicines*, 71(3), 558-563.

Świątek, Ł., Sieniawska, E., Mahomoodally, M.F., Sadeer, N.B., Wojtanowski, K.K., Rajtar, B., Zengin, G., 2021. Phytochemical Profile and Biological Activities of the Extracts from Two *Oenanthe* Species (*O. aquatica* and *O. silaifolia*). *Pharmaceuticals*, 15(1), 50-61.

Tang, W., Harada, K., Kubo, M., Hioki, H., Fukuyama, Y., 2011. Eight new clerodane diterpenoids from the bark of *Ptychopetalum olacoides*. *Natural Product Communications*, 6(3), 327-332.

Tankeo, S.B., Tane, P., Kuete, V., 2015. *In vitro* antibacterial and antibiotic-potentiation activities of the methanol extracts from *Beilschmiedia acuta*, *Clausena anisata*, *Newbouldia laevis* and *Polyscias fulva* against multidrug-resistant Gram-negative bacteria. *BMC Complementary and Alternative Medicine*, 15(1), 412.

Willems, J.L., Khamis, M.M., Saeid, W.M., Purves, R.W., Katselis, G., Low, N.H., El-Aneed, A., 2016. Analysis of a series of chlorogenic acid isomers using differential ion mobility and tandem mass spectrometry. *Analytica Chimica Acta*, 933, 164-174.

Winnie, C.M., Isabel, N.W., Josphat, C.M., 2019. Antibacterial saponins from the leaves of *Polyscias fulva* (Araliaceae). *African Journal of Biotechnology*, 18(19), 390-398.

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The antibiofilm effects of some *Cistus* spp. against pathogenic microorganisms

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ABSTRACT

Recently, the potential antibacterial or antibiofilm effects of some plant species belonging to the *Cistus* sp. have motivated investigation of their use as herbal remedies. In this study, antibiofilm activities of aqueous (dH_2O) leaf extracts of *Cistus laurifolius* L., *C. creticus* L. and *C. salviifolius* L. on some pathogenic microorganisms with biofilm forming ability were investigated. Biofilm forming ability of pathogen test microorganisms were evaluated by congo red agar method and microtiter plate method and all tested microorganisms were confirmed as biofilm producers. *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 11778, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 12600 were evaluated as strong biofilm producers. The highest concentration of examined extracts both showed biofilm inhibition and biofilm eradication against the tested pathogen microorganisms. In particular, studied plant extracts showed good antibiofilm effect, and biofilm eradication against *S. aureus* ATCC 6538 and *S. aureus* ATCC 12600. $MBIC_{50}$ values of *S. aureus* ATCC 6538 were found as 6.25 µg/ml of *C. laurifolius*, and 50 µg/ml of *C. creticus* extracts. Also, 50 µg/ml of *C. creticus* extract showed ≥ 90% inhibition of biofilm growth ($MBIC_{90}$ = 50 µg/ml). $MBEC_{50}$ values of *S. aureus* ATCC 12600 were determined as 6.25 µg/ml in all tested plant extracts and 50 µg/ml of *C. creticus* extract was required to induce ≥ 90% eradication ($MBEC_{90}$) of biofilm growth of *S. aureus* ATCC 12600. Our study revealed that aqueous leaf extracts of *C. laurifolius*, *C. creticus* and *C. salviifolius* could be potential candidates for drug discovery to treat pathogen test microorganisms capable to induce infectious diseases especially by their biofilm forming ability.

1. Introduction

Although the immune defence system of living organisms fighting against pathogenic microorganisms act as a first degree barrier, sometimes, microorganisms overcome this immune system barrier and cause various infections. The treatment of these infections is usually performed using antibiotics but the number of antibiotic-resistant microorganisms has significantly increased recently (Jasovský et al., 2016). Antibiotic resistance is a major problem to public health in which the biofilm forming abilities of some microorganisms contribute to this resistance and, therefore, treatment of infections become more difficult (Olsen, 2015; Richardson, 2017; Aslam et al., 2018; Yan and Bassler, 2019; Bowler et al., 2020). The biofilm is an extracellular matrix that surrounds microbial cells and is comprised of biological polymers such as exopolysaccharide (EPS), protein, and DNA (Leone et al., 2006; Flemming et al., 2016). Various environmental factors are effective in the formation of biofilms such as bacterial strain, surface structure, pH, nutrient amount, and temperature (Donlan and Costerton, 2002). Several researchers have revealed that several pathogen microorganisms show biofilm formation ability which is considered a protective mechanism (Fernandes et al., 2011; Darwish and Asfour, 2013; Silva et al., 2014,

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Raza et al., 2013). Biofilm not only makes microorganisms resistant to adverse environmental conditions, but also protects them from phagocytes and complement systems (Rasmussen Givskov, 2006). Therefore, biofilm-forming microorganisms are considered as the main cause of persistent hospital infections, especially in immunocompromised individual (Roy et al., 2019). Studies have revealed that biofilm-producing bacteria can be 100-10000 times more antibiotic resistant to planktonic forms. (Monzón et al., 2002; Hall-Stoodley et al., 2004; Davies, 2003).

Medicinal and aromatic plants which have various pharmacological properties are important resources in the traditional folk medicine (Ghorbanpour et al., 2017; Ganaie, 2021; Fierascu et al., 2021; Ben Bakrim et al., 2022). Turkey has one of the largest floras in Europe and *Cistus* genus, which is one of the Mediterranean region's characteristic genera, is traditionally used in folk medicine (Catoni et al., 2012; Comandini et al., 2006; Cetin and Yanikoglu, 2006; Attaguile et al., 2000; Ustun and Baykal, 2016). Researchers showed that *Cistus* species are rich in bioactive compounds such as flavonoids, polyphenols, and terpenoids (Stepien et al., 2018; Barrajón-Catalán et al., 2011; Küpeli and Yesilada, 2007; Zalegh et al., 2021). These compounds are effective in using *Cistus* species as anti-inflammatory (Demetzos et al., 2001), antibacterial (Benali et al., 2020; Güvenç et al., 2005), antifungal (Barros et al., 2013), antiviral (Ehrhardt et al., 2007), analgesic (Sayah et al., 2017), and antitumoral (Dimas et al., 2000). Five *Cistus* species belonging to the Cistaceae family grow naturally in Turkey: *C. creticus* L., *C. laurifolius* L., *C. monspeliensis* L., *C. parviflorus* Lam., and *C. salviifolius* L. (Davis, 1988).

This study aimed to determine the antibiofilm activities of aqueous (dH_2O) leaf extracts of *C. laurifolius*, *C. creticus* and *C. salviifolius*, used as herbal remedies in Turkish folk medicine, against some pathogenic test microorganisms. The present study is the first report on antibiofilm effects of *C. laurifolius*, *C. creticus* and *C. salviifolius* against pathogenic test microorganisms.

2. Materials and methods

2.1. Extraction of plant materials

C. laurifolius, was collected from Akdağ-Sandıklı/Afyonkarahisar region (Turkey) and *C. creticus*, *C. salviifolius* were collected from Ağva-Şile/İstanbul region (Turkey) in June-July 2021. These species were identified by Prof. Dr. Mustafa Kargioglu in Afyon Kocatepe University, Faculty of Science and Letters, Department of Molecular Biology and Genetics, using "Flora of Turkey and the East Aegean Islands" (Davis, 1988). The herbarium name and registration numbers of the plants are AKU-10384 for *C. creticus*, AKU-10385 for *C. salviifolius* and AKU-10400 for *C. laurifolius*.

The fresh leaves of *C. laurifolius*, *C. creticus* and *C. salviifolius* were dried in a dryer at 45 °C, then fine powdered in a mill. The modified ultrasonic extraction method was used to prepare dH_2O extracts of *C. laurifolius* (Cl-dH_2O), *C. creticus* (Cc-dH_2O), *C. salviifolius* (Cs-dH_2O). Thirty (30) grams of powdered sample of each extract was prepared with 400 ml each of dH_2O ultrasonicated 1h, at room temperature (Latiff et al., 2021). The available aqueous extract was filtered and the extract was evaporated until dryness in a rotary evaporator (Heidolph) at 40 °C and followed by freeze-drying.

2.2. Pathogen test microorganisms

Listeria monocytogenes ATCC 19115, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* NRRLB 4420, *Enterococcus faecalis* ATCC 51289, *Escherichia coli* ATCC 35218, *Bacillus subtilis* NRS 744, *Staphylococcus aureus* ATCC 6538, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 12600, *P. aeruginosa* ATCC 11778 and *Candida albicans* ATCC 10231 were used as the test microorganisms. The test microorganisms obtained from the bacterial culture collection of Faculty of Pharmacy, Afyonkarahisar Health Sciences University (Afyonkarahisar, Turkey) were used in this study.

2.3. Determination of the biofilm formation of pathogen test microorganisms

Biofilm production of pathogen test microorganisms were evaluated by both congo red agar and microtiter plate methods. All experiments were repeated three times. For congo red agar method, pathogen test microorganisms inoculated to congo red agar medium (brain heart infusion broth 37 g/l, sucrose 50 g/l, congo red 0.8 g/l, and agar 10 g/l) at 37 °C for 24 h, then they were incubated at 25 °C for 48 h (Freeman et al., 1989; Saxena et al., 2014). After incubation periods, the colonies which are black, red, dry, rough, and transparent were evaluated as biofilm positive. Also, the colonies that pinkish red, flat, and central dark colonies were evaluated as biofilm negative (Jain and Agarwal, 2009; Szczuka and Kaznowski, 2014). Then 50 µl of broth culture distributed to a 96-well microtiter plate and re-incubated at 37 °C for 24 hours for microtiter plate method. After incubation period, medium was removed, the wells were washed 3 times by distilled water and 150 µl of crystal violet solution (0.5%, v/v) was transferred into the wells, incubated at 37 °C for 45 min. Then the wells were washed again and 150 µl of ethanol:acetic acid (95:5) was distributed to each well incubated in ambient temperature for 10 min to dissolve the dye. 100 µl of aliquots from each well were transferred to a new microtiter plate and the optical density (OD) of each well was measured at 570 nm using a microplate spectrophotometer (Thermo Scientific Multiskan Sky). The medium was used as a negative control and *P. aeruginosa* ATCC 11778 that is known to generate biofilm was used as the positive control (Kenar et al., 2020). According to the critical OD value (ODc) the biofilm production was scored as: non-biofilm producers [(-), OD ≤ ODc], weak biofilm producers [(+), ODc < OD ≤ 2 x ODc], moderate biofilm producers [(++), 2 x ODc < OD ≤ 4 x ODc], or strong biofilm producers [(+++), OD > 4 x ODc] (Gomes et al., 2019).

2.4. Detection of the EPS production of pathogen test microorganisms

EPS production of pathogen test microorganisms was determined by Marshall and Rawson (1999) method. Broth cultures of pathogen test microorganisms were prepared in NB (Nutrient Broth) and adjusted to 0.5 McFarland turbidity (approximately 1 to 4 x 10^8 cfu/ml) were transferred into a fresh 5 ml NB medium and re-incubated for 20 hours at 37 °C. Then, volume of 1 ml from each culture was transferred into eppendorf tubes and incubated in a water bath at 100 °C for 10-15 minutes. After incubation period, 85% trichloroacetic acid (TCA, 0.17%) were added to the samples and were centrifuged at 14.000 rpm for 20 min. The supernatants were transferred to new eppendorf tubes and the same amount of ethanol was added to each eppendorf tube. Then the eppendorf tubes were centrifuged at 14.000 rpm for 20 min. EPS productions of pathogen test microorganisms were determined by phenol sulfuric acid method. Obtained pellets by centrifugation were dissolved in 100 µl of sterile distilled water and 50 µl of pure phenol was added. Then 500 µl of sulfuric acid was added and vortexed. After incubation for 20 min. at 37 °C, 50 µl of each sample were taken and their absorbance values were determined at 490 nm by a microplate spectrophotometer (Thermo Scientific Multiskan Sky). All

experiments were repeated three times. The results were evaluated according to the glucose standard curve.

2.5. Inhibition of biofilm formation

The biofilm inhibition effects of Cl-dH_2O, Cc-dH_2O, Cs-dH_2O extracts were determined by a 3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide (MTT) colorimetric method of Kairo et al. (1999) with modification of Walencka et al. (2005) and Teanpaisan et al. (2014). Briefly, 100 µl of two-fold serial-diluted extract concentrations (50 to 6.25 µg/ml) were added in 96-well microtiter plates. The fluconazole (10 mg/ml) for *C. albicans* ATCC 10231 and penicillin G (30 mg/ml) for the remaining microorganisms were used as the positive controls, whereas the native medium was used as the negative control. An equal volume of each pathogen test microorganism (1×10^6 cfu/ml) was added and mixed, except in the well with medium alone, and incubated at 37 °C for 24 h. Then supernatants were discarded and washed three times with 150 µl of PBS, and 50 µl of MTT (0.3%) were added and incubated for 2 h at 37 °C. The MTT solution was removed from the wells and 150 µl of DMSO, 25 µl of 0.1 M glycine buffer (pH 10.2) were added to the wells to dissolve the formazan crystals and incubated for 15 min at ambient conditions. The optical density was measured with microplate spectrophotometer (Thermo Scientific Multiskan Sky) at the 570 nm wavelength. The minimal biofilm inhibitory concentration 50% ($MBIC_{50}$) and minimum biofilm inhibitor concentration 90% ($MBIC_{90}$) were calculated. All experiments were repeated three times.

The percentage (%) inhibition was calculated using the equation:

$$[1 - (A_{570} \text{ of the test}/A_{570} \text{ of untreated control})] \times 100$$

2.6. Eradication of biofilm formation

The eradication power of biofilm formation of Cl-dH_2O, Cc-dH_2O, Cs-dH_2O extracts were evaluated using minimum biofilm eradication concentration (MBEC) assay (Teanpaisan et al.,2014). 200 µl (10^6 cfu/ml) of each pathogen test microorganism was inoculated into the 96-well microtiter plate and incubated for 24 h at 37 °C. After the incubation period, the medium was removed and the wells were carefully washed three times with PBS in order to remove non-adherent cells. 200 µl of Cl-dH_2O, Cc-dH_2O, Cs-dH_2O extracts (serial two-fold dilutions from 50 to 6.25 µg/ml) were then added to the wells and incubated for 24 h at 37 °C. Then the adherent bacteria were washed three times PBS and the numbers of surviving microorganisms were determined by an MTT assay. The MBEC value was defined as the concentrations that showed 50% and 90% inhibition of biofilm formation on the medium. The fluconazole (10 mg/ml) for *C. albicans* ATCC 10231 and penicillin G (30 mg/ml) for other bacteria were used as positive controls and the native medium was used as a negative control. All experiments were repeated three times.

The percentage eradication was calculated using the equation of:

$$[1 - (A_{570} \text{ of the test}/A_{570} \text{ of nontreated control})] \times 100$$

3. Results and discussion

3.1. Determination of the biofilm formation of pathogen test microorganisms

In the present study, biofilm generating abilities of some pathogen test microorganisms were demonstrated. According to the congo red agar method, all pathogen test microorganisms in appearances of red, reddish, dry, rough, and transparent colonies were evaluated as biofilm producers. The biofilm forming abilities of pathogen test microorganisms were also evaluated using the microtiter plate method and the results were shown in Table 1. *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 11778, *P. aeruginosa* ATCC 11778, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 12600 were evaluated as strong biofilm producers. *L. monocytogenes* ATCC 19115, *K. pneumoniae* NRRLB 4420, *E. coli* ATCC 35218, and *C. albicans* ATCC 10231 were evaluated as moderate biofilm producers. On the other hand, *E. faecalis* ATCC 51289, *B. subtilis* NRS 744, and *E. coli* ATCC 25922 were evaluated as weak biofilm producers.

Table 1. Biofilm formation of pathogen test microorganisms

Pathogen test microorganisms	Mean OD ± SD	Biofilm formation
L. monocytogenes ATCC 19115	0.74 ± 0.10	++
S. aureus ATCC 25923	1.35 ± 0.15	+++
K. pneumoniae NRRLB 4420	1.03 ± 0.25	++
P. aeruginosa ATCC 11778	1.32 ± 0.18	+++
E. faecalis ATCC 51289	0.34 ± 0.09	+
E. coli ATCC 35218	0.96 ± 0.16	++
B. subtilis NRS 744	0.36 ± 0.03	+
S. aureus ATCC 6538	1.41 ± 0.09	+++
E. coli ATCC 25922	0.41 ± 0.01	+
P. aeruginosa ATCC 27853	1.25 ± 0.11	+++
S. aureus ATCC 12600	1.39 ± 0.17	+++
C. albicans ATCC 10231	0.87 ± 0.04	++

OD: optical density, SD: Standard deviation, +: Weak biofilm producer, ++: Moderate biofilm producer, +++: Strong biofilm producer

Table 2. EPS production by pathogen test microorganisms

Pathogen test microorganisms	EPS (mg/l) ± SD
L. monocytogenes ATCC 19115	6.23 ± 0.45
S. aureus ATCC 25923	6.98 ± 0.23
K. pneumoniae NRRLB 4420	2.27 ± 0.35
P. aeruginosa ATCC 11778	9.15 ± 0.87
E. faecalis ATCC 51289	5.45 ± 0.56
E. coli ATCC 35218	2.20 ± 0.25
B. subtilis NRS 744	1.77 ± 0.78
S. aureus ATCC 6538	7.68 ± 1.12
E. coli ATCC 25922	3.69 ± 0.56
P. aeruginosa ATCC 27853	9.78 ± 0.67
S. aureus ATCC 12600	7.75 ± 0.89
C. albicans ATCC 10231	4.35 ± 0.47

The biofilm formation ability of microorganisms both complicates the treatment of infections and causes serious economic costs in the health and food sectors (Van Houdt and Michiels, 2010; Sabir et al., 2017). Researchers have shown that many microorganisms show biofilm forming ability (Soares et al., 2016; Jamal et al., 2019). In support of our results, Croes et al. (2009) investigated the *in vitro* biofilm formation of clinical *S. aureus* isolates of distinct clonal lineages. This study revealed that *S. aureus* isolates had strong biofilm production ability under specific conditions. The antimicrobial properties and biofilm production of *P. aeruginosa* and *Staphylococcus* spp. strains were also demonstrated by Heidari et al. (2018).

3.2. Detection of the EPS production of pathogen test microorganisms

A glucose standard curve was prepared by using a glucose solution of 10–100 mg/ml to calculate the EPS amounts generated by pathogen test microorganism (Figure 1). EPS production by pathogen test microorganisms are shown in Table 2. The highest EPS production was found in *P. aeruginosa* ATCC 27853 (9.78 ± 0.67 mg/l) and followed by *P. aeruginosa* ATCC 11778 (9.15 ± 0.87 mg/l). The lowest EPS production was determined in *B. subtilis* NRS 744 (1.77 ± 0.78). Wu et al. (2020), evaluated the biofilm formation by some pathogen microorganisms and reported that some of these pathogens, with biofilm forming ability, showed resistance against various antibiotics used. Researchers revealed that the produced EPS were responsible for the cohesion of microorganisms, adhesion of biofilms to surfaces and also acted as adhesives between cells (Costa et al., 2018).

3.3. Inhibition and eradication of biofilm formation

The concentrations of Cl-dH_2O, Cs-dH_2O and Cc-dH_2O extracts required to inhibit ≥ 50% biofilm formation of pathogen test microorganisms are shown in Figures 2, 3 and 4. $MBIC_{50}$ values of Cl-dH_2O and Cs-dH_2O extracts on *S. aureus* ATCC 6538 were found as 6.25 and 50 µg/ml, respectively. Also, 50 µg/ml of Cc-dH_2O extract was required to inhibit ≥ 90% of biofilm growth ($MBIC_{90}$ = 50 µg/ml). $MBIC_{50}$ values of *E. coli* ATCC 25922 were found as 12.5 µg/ml for both Cl-dH_2O and Cs-dH_2O, and 25 µg/ml for Cc-dH_2O extracts. The volume of Cl-dH_2O and Cc-dH_2O extracts required to inhibit ≥ 50% of *S. aureus* ATCC 12600 biofilm formation ($MBIC_{50}$) was found as 50 µg/ml. On the other hand, $MBIC_{50}$ of the Cl-dH_2O extract on *C. albicans* ATCC 10231 was determined as 50 µg/ml. Biofilm inhibition of pathogen test microorganisms by positive controls are shown in Figure 5.

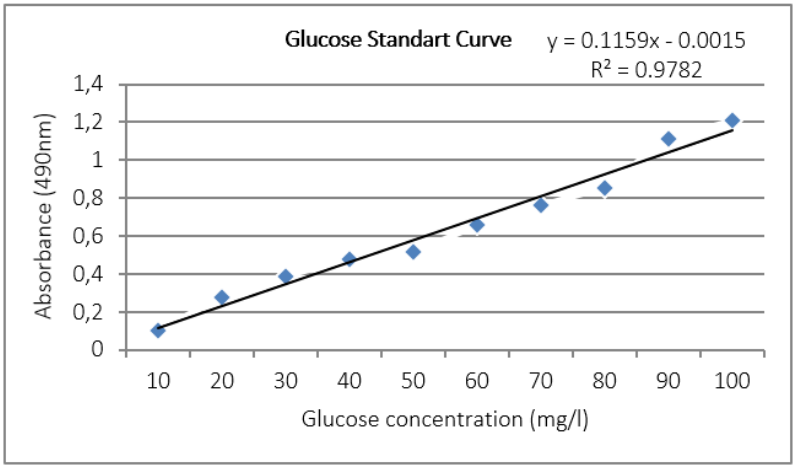

Figure 1. Glucose standart curve

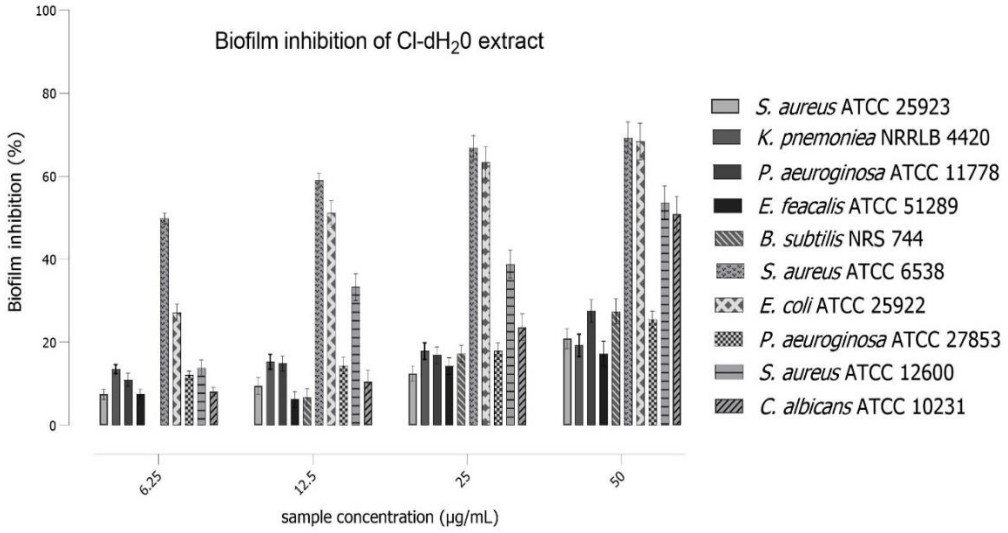

Figure 2. Biofilm inhibition of pathogen test microorganism by Cl-dH_2O extract

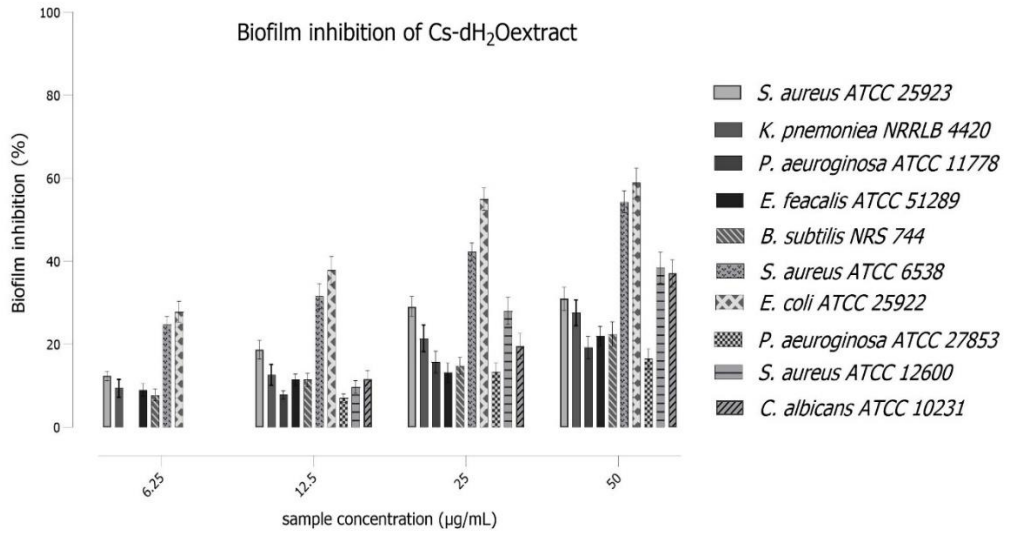

Figure 3. Biofilm inhibition of pathogen test microorganisms by Cs-dH$_2$O extract

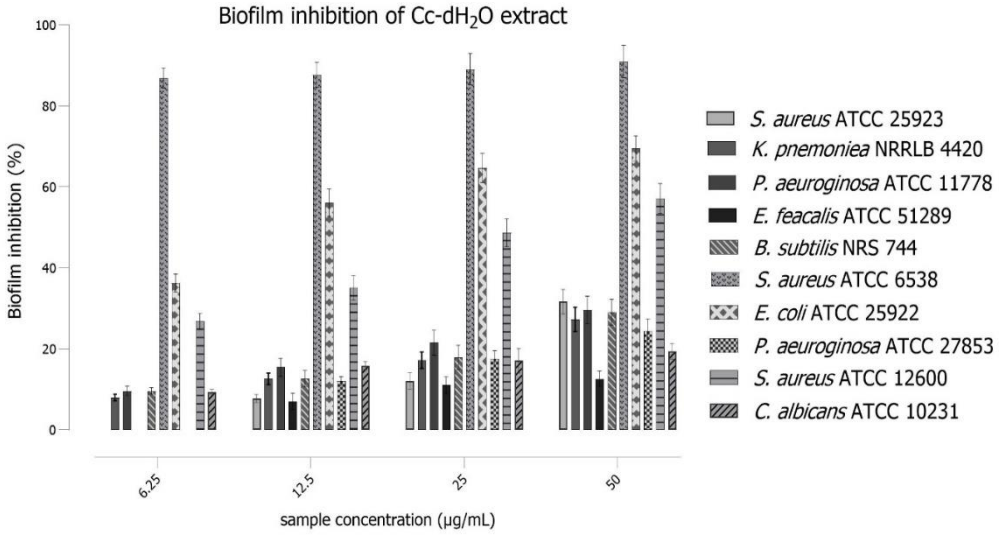

Figure 4. Biofilm inhibition of pathogen test microorganisms by Cc-dH$_2$O extract

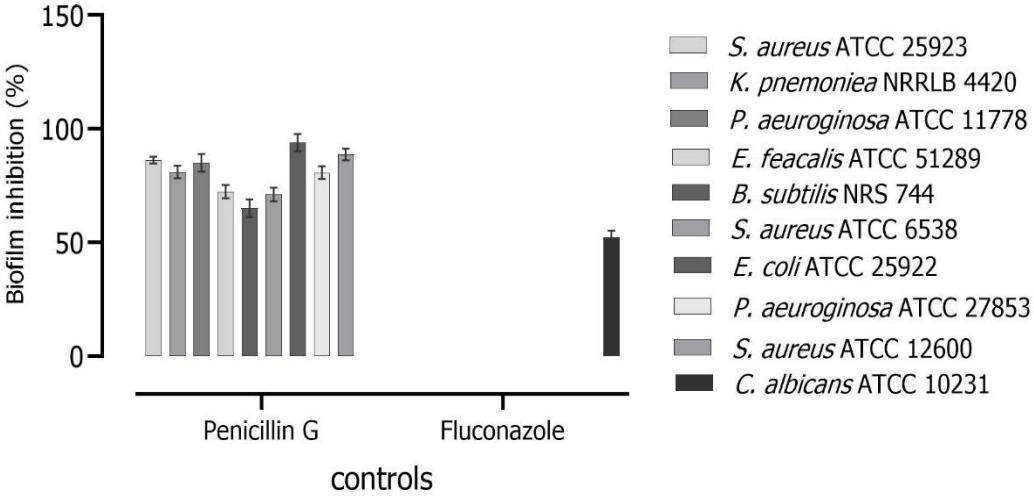

Figure 5. Biofilm inhibition of pathogen test microorganisms by positive controls

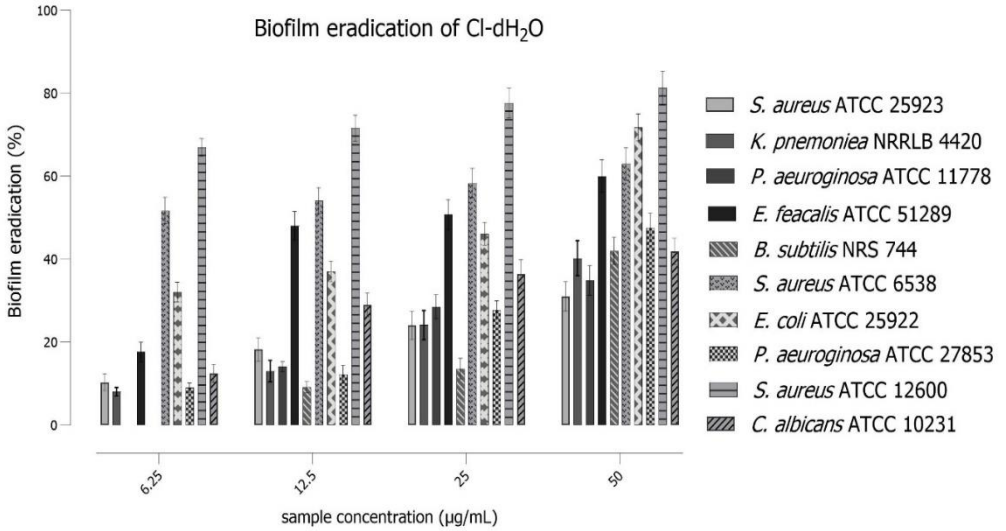

Figure 6. Biofilm eradication of pathogen test microorganisms by Cl-dH_2O extract

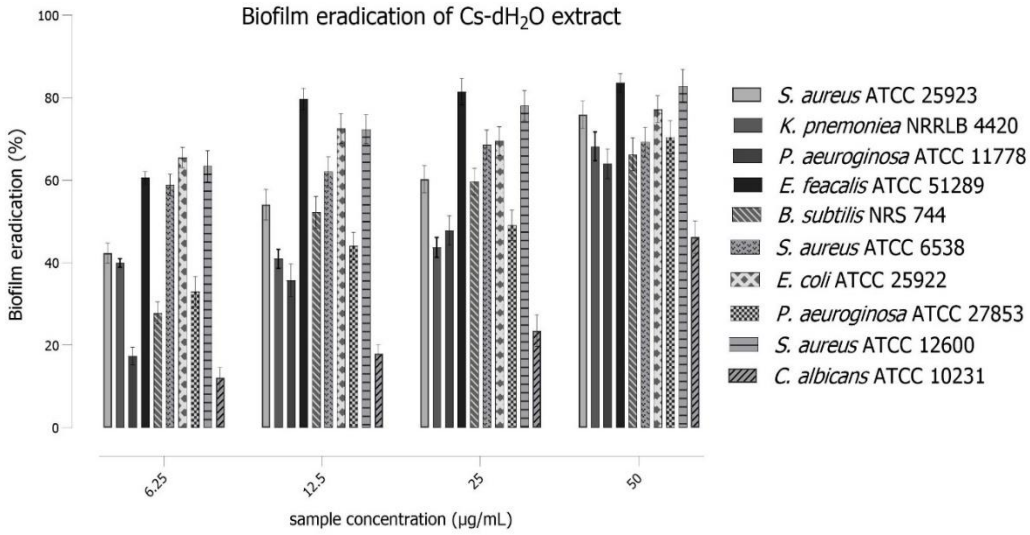

Figure 7. Biofilm eradication of pathogen test microorganisms by Cs-dH_2O extract

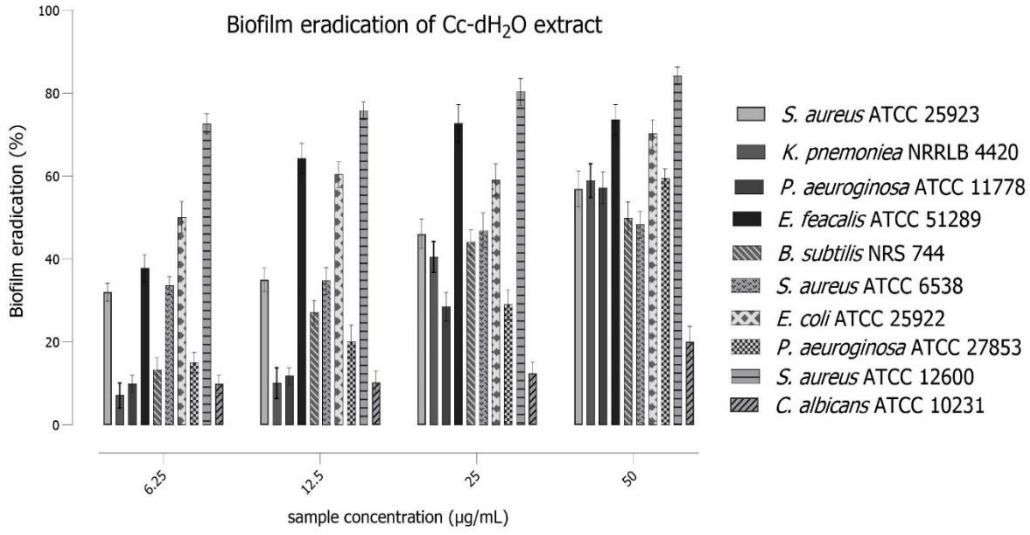

Figure 8. Biofilm eradication of pathogen test microorganisms by Cc-dH_2O extract

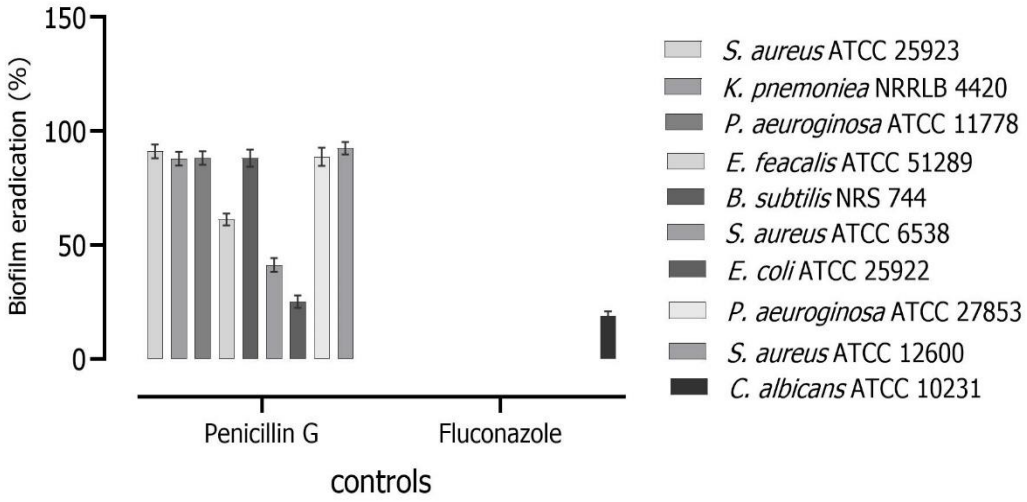

Figure 9. Biofilm eradication of pathogen test microorganisms by positive controls

The eradication of the biofilm formation of pathogen test microorganisms by Cl-dH$_2$O, Cc-dH$_2$O and Cs-dH$_2$O at various concentrations is demonstrated in Figures 6, 7 and 8. The concentrations of Cs-dH$_2$O and Cc-dH$_2$O extracts to eradicate ≥ 50% biofilm formation (MBEC$_{50}$) of *S. aureus* ATCC 25923 were found as 12.5 and 50 µg/ml, respectively. MBEC$_{50}$ values of Cs-dH$_2$O and Cc-dH$_2$O extracts on *K. pneumoniae* NRRLB 4420 strain were determined as 50 µg/ml. MBEC$_{50}$ values of Cs-dH$_2$O and Cc-dH$_2$O extracts on *P. aeruginosa* ATCC 11778 were found as 25 µg/ml and 50 µg/ml, respectively. The amounts of Cl-dH$_2$O, Cc-dH$_2$O and Cs-dH$_2$O extracts required to inhibit preformed biofilm formation by ≥ 50% (MBEC$_{50}$) in *E. faecalis* ATCC 51289 strain were determined as 25.0, 12.5 and 6.25 µg/ml, respectively. MBEC$_{50}$ values of Cc-dH$_2$O and Cs-dH$_2$O on *B. subtilis* NRS 744 were found as 50 µg/ml and 12.5 µg/ml, respectively. MBEC$_{50}$ of Cl-dH$_2$O and Cs-dH$_2$O on *S. aureus* ATCC 6538 were both determined as 6.25 µg/ml, whereas it was found 50 µg/ml for the Cc-dH$_2$O extract. MBEC$_{50}$ values of Cs-dH$_2$O and Cc-dH$_2$O extracts on *E. coli* ATCC 25922 were both found as 6.25 µg/ml. MBEC$_{50}$ values of Cl-dH$_2$O and Cc-dH$_2$O on *P. aeruginosa* ATCC 27853 were both determined as 50 µg/ml, whereas it was calculated as 25 µg/ml for Cs-dH$_2$O extract. Moreover, MBEC$_{50}$ values of all Cl-dH$_2$O, Cc-dH$_2$O and Cs-dH$_2$O extracts on *S. aureus* ATCC 12600 were determined as 6.25 µg/ml. Also, 50 µg/ml of Cc-dH$_2$O extract was sufficient to inhibit ≥ 90% of the preformed biofilm formation (MBEC$_{90}$) of *S. aureus* ATCC 12600. Finally, the MBEC$_{50}$ of Cs-dH$_2$O extract on *C. albicans* ATCC 10231 strain was found as 50 µg/ml. Biofilm eradication levels of pathogen test microorganisms by positive controls are shown in Figure 9.

In this study, the antibiofilm effects of Cl-dH$_2$O, Cc-dH$_2$O and Cs-dH$_2$O leaf extracts were evaluated against some pathogenic test microorganisms. The results of the present study clearly demonstrated that the percentages of biofilm inhibition and biofilm eradication of pathogen test microorganisms increased depending on the increasing concentrations of Cl-dH$_2$O, Cc-dH$_2$O and Cs-dH$_2$O leaf extracts. The highest concentration of Cl-dH$_2$O, Cc-dH$_2$O and Cs-dH$_2$O extracts were effective as MBIC$_{50}$ and MBEC$_{50}$ of positive controls applied to pathogen test microorganisms. Although numerous studies have been conducted to evaluate biological effects of *Cistus* sp., there is limited research on antibiofilm activity of *Cistus* sp. against pathogen microorganisms (Zalegh et al., 2021). Hannig et al. (2008) revealed that *Cistus*-tea may be used to reduce the initial bacterial adhesion. Lekbach et al. (2018) showed that *C. ladanifer* extract inhibited the growth of *P. aeruginosa* planktonic cells and the biofilm formation. In another study, Álvarez-Martínez et al. (2021) revealed that *C. salviifolius* extract exhibited higher antimicrobial activity against *S. aureus* isolates. Previous studies have demonstrated a relationship between polyphenols of plant extracts and their antimicrobial activities (Zalegh et al., 2021). In a previous study, it was suggested that the antibiofilm activities of Cl-dH$_2$O, Cc-dH$_2$O and Cs-dH$_2$O leaf extracts against pathogen test microorganisms could be associated with bioactive substances present in these extracts.

4. Conclusions

The results of this study revealed that the plant species that examined in this study showed varying degrees of antibiofilm activities against *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *K. pneumoniae* NRRLB 4420, *E. faecalis* ATCC 51289, *E. coli* ATCC 35218, *B. subtilis* NRS 744, *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 12600, *P. aeruginosa* ATCC 11778, and *C. albicans* ATCC 10231. The percentage of biofilm eradication values of these extracts were more effective than the biofilm inhibition values against the pathogen test microorganisms. Among the examined extracts, Cs-dH$_2$O showed greater antibiofilm effect against tested pathogen microorganisms. Therefore, Cs-dH$_2$O leaf extracts could be a potential candidate for drug discovery, particularly in the treatment of immune deficient patients. Also, these plant extracts may be potential candidates for further investigation to isolate antimicrobial compounds and to determine the mechanism of activity.

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None.

Conflict of interest

The authors declare no confict of interest in conducting and reporting this study.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

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Supplementary File

None.

References

Álvarez-Martínez, F.J., Rodríguez, J.C., Borrás-Rocher, F., Barrajón-Catalán, E., Micol, V., 2021. The antimicrobial capacity of *Cistus salviifolius* and *Punica granatum* plant extracts against clinical pathogens is related to their polyphenolic composition. *Scientific Reports*, 11(1), 1-12.

Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., Baloch, Z., 2018. Antibiotic resistance: a rundown of a global crisis. *Infection and Drug Resistance*, 11, 1645-1658.

Attaguile, G., Russo, A., Campisi, A., Savoca, F., Acquaviva, R., Ragusa, N., Vanella, A., 2000. Antioxidant activity and protective effect on DNA cleavage of extracts from *Cistus incanus* L. and *Cistus monspeliensis* L. *Cell Biology and Toxicology*, 16(2), 83-90.

Barrajón-Catalán, E., Fernández-Arroyo, S., Roldán, C., Guillén, E., Saura, D., Segura-Carretero, A., Micol, V., 2011. A systematic study of the polyphenolic composition of aqueous extracts deriving from several *Cistus* genus species: evolutionary relationship. *Phytochemical Analysis*, 22(4), 303-312.

Barros, L., Dueñas, M., Alves, C.T., Silva, S., Henriques, M., Santos-Buelga, C., Ferreira, I.C., 2013. Antifungal activity and detailed chemical characterization of *Cistus ladanifer* phenolic extracts. *Industrial Crops and Products*, 41, 41-45.

Ben Bakrim, W., Aghraz, A., Hriouch, F., Larhsini, M., Markouk, M., Bekkouche, K., Dugo, G., 2022. Phytochemical study and antioxidant activity of the most used medicinal and aromatic plants in Morocco. *Journal of Essential Oil Research*, 34(2), 131-142.

Benali, T., Bouyahya, A., Habbadi, K., Zengin, G., Khabbach, A., Hammani, K., 2020. Chemical composition and antibacterial activity of the essential oil and extracts of *Cistus ladaniferus* subsp. *ladanifer* and *Mentha suaveolens* against phytopathogenic bacteria and their ecofriendly management of phytopathogenic bacteria. *Biocatalysis and Agricultural Biotechnology*, 28, 101696.

Bowler, P., Murphy, C., Wolcott, R., 2020. Biofilm exacerbates antibiotic resistance: Is this a current oversight in antimicrobial stewardship?. *Antimicrobial Resistance & Infection Control*, 9(1), 1-5.

Catoni, R., Gratani, L., Varone, L., 2012. Physiological, morphological and anatomical trait variations between winter and summer leaves of *Cistus* species. *Flora-Morphology, Distribution, Functional Ecology of Plants*, 207(6), 442-449.

Cetin, H., Yanikoglu, A., 2006. A study of the larvicidal activity of *Origanum* (Labiatae) species from southwest Turkey. *Journal of Vector Ecology*, 31(1), 118-122.

Comandini, O., Contu, M., Rinaldi, A.C., 2006. An overview of *Cistus* ectomycorrhizal fungi. *Mycorrhiza*, 16(6), 381-395.

Costa, O.Y., Raaijmakers, J.M., Kuramae, E.E., 2018. Microbial extracellular polymeric substances: ecological function and impact on soil aggregation. *Frontiers in Microbiology*, 9, 1636.

Croes, S., Deurenberg, R.H., Boumans, M.L.L., Beisser, P.S., Neef, C., Stobberingh, E.E., 2009. *Staphylococcus aureus* biofilm formation at the physiologic glucose concentration depends on the *S. aureus* lineage. *BMC Microbiology*, 9(1), 1-9.

Darwish, S.F., Asfour, H.A., 2013. Investigation of biofilm forming ability in *Staphylococci* causing bovine mastitis using phenotypic and genotypic assays. *The Scientific World Journal*, 2013, 378492.

Davies, D., 2003. Understanding biofilm resistance to antibacterial agents. *Nature Reviews Drug Discovery*, 2(2), 114-122.

Davis, P.H., 1988: Flora of Turkey and the East Aegean Islands. Vol. 10. Edinburgh University Press, p. 61–62.

Demetzos, C., Dimas, K., Hatziantoniou, S., Anastasaki, T., Angelopoulou, D., 2001. Cytotoxic and anti-inflammatory activity of labdane and *cis*-clerodane type diterpenes. *Planta Medica*, 67(07), 614-618.

Dimas, K., Demetzos, C., Angelopoulou, D., Kolokouris, A., Mavromoustakos, T., 2000. Biological activity of myricetin and its derivatives against human leukemic cell lines in vitro. *Pharmacological Research*, 42(5), 475-478.

Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15(2), 167-193.

Ehrhardt, C., Hrincius, E.R., Korte, V., Mazur, I., Droebner, K., Poetter, A., Ludwig, S., 2007. A polyphenol rich plant extract, CYSTUS052, exerts anti influenza virus activity in cell culture without toxic side effects or the tendency to induce viral resistance. *Antiviral Research*, 76(1), 38-47.

Fernandes, J.B.C., Zanardo, L.G., Galvão, N.N., Carvalho, I.A., Nero, L.A., Moreira, M.A.S., 2011. *Escherichia coli* from clinical mastitis: serotypes and virulence factors. *Journal of Veterinary Diagnostic Investigation*, 23(6), 1146-1152.

Fierascu, R.C., Fierascu, I., Baroi, A.M., Ortan, A., 2021. Selected aspects related to medicinal and aromatic plants as alternative sources of bioactive compounds. *International Journal of Molecular Sciences*, 22(4), 1521.

Flemming, H.C., Wingender, J., Szewwzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, 14,563-575.

Freeman, D.J., Falkiner, F.R., Keane, C.T., 1989. New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology*, 42(8), 872-874.

Ganaie, H.A., 2021. Review of the active principles of medicinal and aromatic plants and their disease fighting properties. In Medicinal and aromatic plants (pp. 1-36). Academic Press.

Ghorbanpour, M., Hadian, J., Nikabadi, S., Varma, A., 2017. Importance of medicinal and aromatic plants in human life. In Medicinal Plants and Environmental Challenges (pp. 1-23). Springer, Cham.

Gomes, F., Martins, N., Ferreira, I.C., Henriques, M., 2019. Anti-biofilm activity of hydromethanolic plant extracts against *Staphylococcus aureus* isolates from bovine mastitis. *Heliyon*, 5(5), e01728.

Güvenç, A., Yıldız, S., Özkan, A.M., Erdurak, C.S., Coşkun, M., Yılmaz, G., Okada, Y., 2005. Antimicrobiological studies on turkish *Cistus* species. *Pharmaceutical Biology*, 43(2), 178-183.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology*, 2(2), 95-108.

Hannig, C., Spitzmüller, B., Al-Ahmad, A., Hannig, M., 2008. Effects of *Cistus*-tea on bacterial colonization and enzyme activities of the *in situ* pellicle. *Journal of Dentistry*, 36(7), 540-545.

Heidari H.M., Ebrahim-Saraie H.S., Mirzaei A., Taji, A., Hosseini S.R., Motamedifar, M., 2018. Characterization of virulence factors, antimicrobial resistance patterns and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus* spp. strains isolated from corneal infection. *Journal Français d'Ophtalmologie*, 41, 9823-829.

Jain, A., Agarwal, A., 2009. Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. *Journal of Microbiological Methods*, 76(1), 88-92.

Jamal, M., Andleeb, S., Jalil, F., Imran, M., Nawaz, M.A., Hussain, T., Das, C.R., 2019. Isolation, characterization and efficacy of phage MJ2 against biofilm forming multi-drug resistant *Enterobacter cloacae*. *Folia Microbiologica*, 64(1), 101-111.

Jasovský, D., Littmann, J., Zorzet, A., Cars, O., 2016. Antimicrobial resistance—a threat to the world's sustainable development. *Upsala Journal of Medical Sciences*, 121(3), 159-164.

Kairo, S.K., Bedwell, J., Tyler, P.C., Carter, A., Corbel, M.J., 1999. Development of a tetrazolium salt assay for rapid determination of viability of BCG vaccines. *Vaccine*, 17(19), 2423-2428.

Kenar, B., Erik, M., Erdoğmuş, S.F., Korcan, S.E., Köse, Z., Durmaz, G., 2020. The determination of antimicrobial and antibiofilm activities of foodborne lactic acid bacteria against *Enterobacter cloacae* isolates. *Turkish Journal of Veterinary & Animal Sciences*, 44(1), 59-68.

Küpeli, E., Yesilada, E., 2007. Flavonoids with anti-inflammatory and antinociceptive activity from *Cistus laurifolius* L. leaves through bioassay-guided procedures. *Journal of Ethnopharmacology*, 112(3), 524-530.

Latiff, N.A., Ong, P.Y., Abd Rashid, S.N.A., Abdullah, L.C., Mohd Amin, N.A., Fauzi, N.A.M., 2021. Enhancing recovery of bioactive compounds from *Cosmos caudatus* leaves via ultrasonic extraction. *Scientific Reports*, 11(1), 17297.

Lekbach, Y., Xu, D., El Abed, S., Dong, Y., Liu, D., Khan, M.S., Yang, K., 2018. Mitigation of microbiologically influenced corrosion of 304L stainless steel in the presence of *Pseudomonas aeruginosa* by *Cistus ladanifer* leaves extract. *International Biodeterioration & Biodegradation*, 133, 159-169.

Leone, S., Molinaro, A., Alfieri, F., Cafaro, V., Lanzetta, R., Di Donato, A., Parrilli, M., 2006. The biofilm matrix of *Pseudomonas* sp. OX1 grown on phenol is mainly constituted by alginate oligosaccharides. *Carbohydrate Research*, 341(14), 2456-2461.

Marshall, V.M., Rawson, H.L., 1999. Effects of exopolysaccharide-producing strains of thermophilic lactic acid bacteria on the texture of stirred yoghurt. *International Journal of Food Science & Technology*, 34(2), 137-143.

Monzón, M., Oteiza, C., Leiva, J., Lamata, M., Amorena, B., 2002. Biofilm testing of *Staphylococcus epidermidis* clinical isolates: low performance of vancomycin in

relation to other antibiotics. *Diagnostic Microbiology and Infectious Disease*, 44(4), 319-324.
Olsen, I., 2015. Biofilm-specific antibiotic tolerance and resistance. *European Journal of Clinical Microbiology & Infectious Diseases*, 34(5), 877-886.
Rasmussen, T.B., Givskov, M., 2006. Quorum sensing inhibitors: a bargain of effects. *Microbiology*, 152(4), 895-904.
Raza, A., Muhammad, G., Sharif, S., Atta, A., 2013. Biofilm producing *Staphylococcus aureus* and bovine mastitis: a review. *Molecular Microbiology Research*, 3(1), 1-18.
Richardson, L.A., 2017. Understanding and overcoming antibiotic resistance. *PLoS Biology*, 15(8), e2003775.
Roy, R., Tiwari, M., Donelli, G., Tiwari, V., 2019. Strategies for Combating Bacterial Biofilms: A Focus on Anti-Biofilm Agents and Their Mechanisms of Action. *Virulence*, 9(1), 522–554.
Sabir, N., Ikram, A., Zaman, G., Satti, L., Gardezi, A., Ahmed, A., Ahmed, P., 2017. Bacterial biofilm-based catheter-associated urinary tract infections: Causative pathogens and antibiotic resistance. *American Journal of Infection Control*, 45(10), 1101-1105.
Saxena, N., Maheshwari, D., Dadhich, D., Singh, S., 2014. Evaluation of Congo red agar for detection of biofilm production by various clinical *Candida* isolates. *Journal of Evolution of Medical and Dental Sciences*, 3(59), 13234-13239.
Sayah, K., Chemlal, L., Marmouzi, I., El Jemli, M., Cherrah, Y., Faouzi, M.E.A., 2017. *In vivo* anti-inflammatory and analgesic activities of *Cistus salviifolius* (L.) and *Cistus monspeliensis* (L.) aqueous extracts. *South African Journal of Botany*, 113, 160-163.
Silva, V.O., Soares, L.O., Silva Júnior, A., Mantovani, H.C., Chang, Y.F., Moreira, M.A.S., 2014. Biofilm formation on biotic and abiotic surfaces in the presence of antimicrobials by *Escherichia coli* isolates from cases of bovine mastitis. *Applied and Environmental Microbiology*, 80(19), 6136-6145.
Soares, G.G., Costa, J.F., Melo, F., Mola, R., Balbino, T.C.L., 2016. Biofilm production and resistance profile of *Enterobacter* sp. strains isolated from pressure ulcers in Petrolina, Pernambuco, Brazil. *Jornal Brasileiro de Patologia e Medicina Laboratorial*, 52, 293-298.
Stepien, A., Aebisher, D., Bartusik-Aebisher, D., 2018. Biological properties of *Cistus* species. *European Journal of Clinical and Experimental Medicine*, 16, 127-132.
Szczuka, E., Kaznowski, A., 2014. Antimicrobial activity of tigecycline alone or in combination with rifampin against *Staphylococcus epidermidis* in biofilm. *Folia Microbiologica*, 59(4), 283-288.
Teanpaisan, R., Senapong, S., Puripattanavong, J., 2014. *In vitro* antimicrobial and antibiofilm activity of *Artocarpus lakoocha* (Moraceae) extract against some oral pathogens. *Tropical Journal of Pharmaceutical Research*, 13(7), 1149-1155.
Ustun, O., Baykal, T., 2016. Bioactivities of ethanolic extract and its fractions of *Cistus laurifolius* L. (Cistaceae) and *Salvia wiedemannii* Boiss. (Lamiaceae) species. *Pharmacognosy Magazine*, 12(Suppl 1), S82-S85.
Van Houdt, R., Michiels, C.W., 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology*, 109(4), 1117-1131.
Walencka, E., Sadowska, B., Ro´zalska, S., Hryniewicz, W., Rozalska, B., 2005. Lysostaphin as a potential therapeutic agent for staphylococcal biofilm eradication. *Polish Journal of Microbiology*, 54, 191-200.
Wu, X., Al Farraj, D.A., Rajaselvam, J., Alkufeidy, R.M., Vijayaraghavan, P., Alkubaisi, N.A., Alshammari, M.K., 2020. Characterization of biofilm formed by multidrug resistant *Pseudomonas aeruginosa* DC-17 isolated from dental caries. *Saudi Journal of Biological Sciences*, 27(11), 2955-2960.
Yan, J., Bassler, B.L., 2019. Surviving as a community: antibiotic tolerance and persistence in bacterial biofilms. *Cell Host & Microbe*, 26(1), 15-21.
Zalegh, I., Akssira, M., Bourhia, M., Mellouki, F., Rhallabi, N., Salamatullah, A.M., Mhand, R.A., 2021. A review on *Cistus* sp.: Phytochemical and antimicrobial activities. *Plants*, 10(6), 1214.

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REVIEW

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Fungi mediated agarwood (*A. malaccensis*) production and their pharmaceutical applications: A systematic review

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ABSTRACT

Aquilaria malaccensis is a tropical tree that produces expensive resinous heartwood agarwood through the natural process induced by natural or artificial injury or microbial infection. Fungi are commonly noticed as the main microbial component responsible for agarwood formation. The current review investigated the agarwood quality and fungi diversity in artificial and natural agarwood from *A. malecensis* trees from the rainforest for various pharmaceutical applications. Apart from being an aromatherapy material, in medicinal prospect, agarwood can be used as a carminative, stimulant for heart palpitation, tonic during pregnancy, remedy during the post-natal recovery period, and cure for the disease of the female genital part. The whitebark of agarwood is believed to heal jaundice and body pain. Moreover, agarwood helps to relieve body pain, warm the abdomen, relieve asthma, treat coughs, and acroparalysis acts as antihistamine, analgesic and anti-inflammatory. In Chinese traditional medicine, it is helpful as a sedative to relieve gastric problems, relieve rheumatism and high fever. Agarwood properties were able to fight cancer cells but very little is known about this process. The present review also illustrated the variety of kinds of pharmaceutical applications in the treatment of various diseases. Current review findings proved that artificial agarwood may produce quality equal to natural agarwood and may not be affected by fungi interacting with the tree, which can be used as a superior pharmaceutical application.

1. Introduction

Malaysia's rainforest is one of the countries rich in its biodiversity and hosts over 500.000 plant species that have been exploited since ancient times for their high economic value (Elias et al., 2017). *Aquilaria* species or *Karas* trees come from the Thymelaeaceae family and are well distributed throughout peninsular Malaysia. This plant species is widely known for its gaharu; a scented wood or agarwood/sandalwood. Agarwood, the fragrant resin-infused wood derived from the wounded trees of *Aquilaria* species, is categorized as a non-timber product and is highly valued in West Asia and the Middle East (Zhang et al., 2010; Ramli et al., 2022). *Aquilaria malaccensis* is the most popular source of agarwood among the *Aquilaria* species and has become the primary agarwood producer in Malaysia. Agarwood contains a unique property by emitting a wonderful fragrant when this dark resinous wood is burnt. As a result, agarwood is widely utilized as an essential component in perfumes and incenses production. In addition, it has also been used in traditional medicines for centuries. It recently has been incorporated into pharmaceutical products to treat many illnesses, including coughs, acroparalysis, and asthma, and has been used as an antihistamine (Mohamed et al., 2014).

Agarwood's high demand and selling price have seriously affected the natural sources of *A. malaccensis* resulting from inappropriate harvesting. Currently, *A. malaccensis* is listed as an endangered species in the Convention on International Trade in Endangered Species of Wild Fauna and Flora. Growing agarwood using an optimum and sustainable method would permit the increasing world market demand for medicines, perfumes, art-crafts, and others to be met. Nowadays, *Aquilaria* trees are being cultivated on a large scale, and many investigations have been performed into

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formulating the best artificial inoculation methods for agarwood induction in plantation trees (Bhuiyan et al., 2009; Mohamed et al., 2014). Both forms of reforestation of *A. malaccensis* and artificial agarwood-inducing methods will guarantee a continuous supply of agarwood and can conserve the wild *A. malaccensis* species.

In natural rainforests, agarwood formation in *A. malaccensis* happens randomly and usually at a low level. The analysis predicted that the trees which develop agarwood due to natural infections only range from 7-10% (Ng et al., 1997). Generally, fungi are considered the significant microbial component responsible for agarwood formation. From the observation, healthy trees never produce fragrant resin. Agarwood formation is mainly attributed to trees' biotic and abiotic stress exposure (which can be physically or chemically), which activates their defense mechanism (Novriyanti et al., 2010). Fungi identification in natural agarwood has been investigated previously, and several species were discovered to be either endophyte or pathogenic. It is known that wounding and microbial infection effectively produce resin in agarwood. However, none of the fungi found was proven to induce artificial agarwood to produce quality similar to nature agarwood. Perhaps, a different fungi community makes the difference. To solve the intersection of fungi species works in nature, agarwood and artificial agarwood would be a tremendous success to ensure continuous superior agarwood supply.

Continuous demand and shortage supply of natural agarwood lead to the production of artificial agarwood. However, the quality of artificial agarwood is arguable as it is not even close to natural agarwood quality. The mechanism of high-quality agarwood formation remains a mystery despite fungal discovery from agarwood samples. Fungal interaction proves a relationship in inducing resin synthesis in agarwood trees. Various species of fungi have been discovered from agarwood and even applied as inoculants, but the agarwood is either low quality or inconsistent. The difference between natural and artificial agarwood may be caused by the different diversity of fungi in both agarwood trees. Thus, comparing these two samples regarding agarwood quality and fungi diversity is necessary to determine an effective fungi group as agarwood inducers. Moreover, the comparison may prove if artificial quality can be equal to nature agarwood based on fungal infection. This review analyses agarwood quality samples from natural and artificial agarwood followed by fungal identification based on morphology observation and molecular approach for future pharmaceutical applications.

2. *Aquilaria* species

Aquilaria spp. and Gyrinops from the botanical family Thymelaeaceae naturally produce distinct fragrance trees. *Aquilaria* spp. is common in tropical climates, as in Asian countries and Papua Guinea. However, not all *Aquilaria* spp. produce resinous wood (Sumarna, 2005; Wiriadinata et al., 2010). *Aquilaria* spp. is distributed from Southeast Asia to China, India, and Papua (New Guinea). Each country has different species of *Aquilaria*. *Aquilaria* spp. prefers to grow in the dipterocarp or mixed dipterocarp forest. It is usually populated at up to 1000 m sea level and from 270 m to 500 m within the temperature of 22 °C to 22 °C (Chua, 2008). However, it may adapt to the plantation of rocky, sandy, or calcareous soil areas equipped with well-drained slopes and ridges (Chakrabarti et al., 1994; Sumadiwangsa, 1997). *Aquilaria* species such as *A. malaccensis*, *A. cumingiana* and *A. microcarpa* were common in Malaysia and Indonesia. Meanwhile, *A. crassna* is found in Malaysia, Thailand, Cambodia, and Vietnam. *A. hirta*, can be found in the forests of Malaysia, Indonesia and Thailand. *A. achalloga* and *A. khasiana* can only be found in India. *A. acuminata* is known in Indonesia, Philippines, and Papua (New Guinea). *A. baillonil* is native to Thailand and Cambodia. Phillipine and Papua (New Guinea) both own *A. filaria* species. Some *Aquilaria* species are endemic in certain countries, such as *A. brachyantha* and *A. rostrata* which are endemic to Malaysia, while *A. sinensis* can only be found in the China region, *A. beccariana* is grown in Indonesia, *A. apiculina* in Phillipine and *A. baneonsis* in Vietnam (Akter et al., 2013).

2.1. Aquilaria malaccensis

A. malaccensis is a tree of about 20-40 m in height and diameter at breast height (dbh) of 60 cm (Duriyaprapan et al., 2003). *A. malaccensis* is a non-timber that appears white, light in weight and soft in density. Leaves of *A. malaccensis* are alternate and elliptic, about 3-5 cm wide and 6-10 cm long, with 12-16 pairs of veins. *Aquilaria* tree would grow for about a hundred years if undisturbed in the forest and start to produce small, white flowers as early as four years old, depending on the species (Akter et al., 2013), but only able to produce seeds between seven to nine years old (Chua, 2008). Its inflorescence is described as a terminal or axillary umbel, containing flowers 5 mm long, yellowish green or white (Duriyaprapan et al., 2003). The juvenile fruit is a green egg-shaped capsule 4 cm long and 2.5 cm wide, with a pubescent leathery exocarp. The mature fruits are blackish brown and can be collected directly from the tree, each containing two seeds (Duriyaprapan et al., 2003). The juvenile fruit of the *Aquilaria* tree appears in an oval shape of 4 cm in length and 2.5 cm in width with a hairy surface. Each fruit contains two seeds (Duriyaprapan et al., 2003). In the forest, the seeds will casted around the mother tree. Seed viability is within one week after seeding mature. Seed germination takes 16 to 63 days, depending on the sowing time after seed maturity. Germination is at the highest rate if the seed is sown immediately (Ng et al., 1997). This may be due to changes in enzymes and hormone accumulation in the seed that control the germination process. Although its fruit is recalcitrant without human interference, seedlings found in the forest hardly grow on commercial plantations after being transplanted. Mature trees can grow up to 40 m in height and reach 2.5 cm dbh with a growth rate of approximately 0 to 1.95 cm per year (Sumadiwangsa, 1997).

2.2. Agarwood production in A. malaccensis

Agarwood production from *A. malaccensis* has been recorded through the discovery of aromatic compounds or terpene family compounds. Wu et al. (2012) found four new sesquiterpenoids and new 2-(2-phenylethyl)-4H-chromone-4-one [-2-(2-phenylethyl)-4H-1-benzopyran-4-one] derivative from agarwood sample of *A. malaccensis*. While mature *A. malaccensis* can produce agarwood, the young tree has been proven to synthesize agarwood compounds such as benzylacetone, anisylacetone, guaiene, and palustrol when infected with fungus (Mohamed et al., 2014). In addition, matured agarwood from wild habitat may produce agarospirol, alloalromadendrene α-elemol, γ-eudesmol, and guaiol when analyzed with gas chromatography-mass spectrometry (GC-MS) (Tsan and Mohamed, 2014). Many previous studies showed that *A. malaccensis* could produce aromatic agarwood when burned or analyzed with a spectrometry instrument (Chen et al., 2011; Jayachandran et al., 2014; Mei et al., 2013).

3. Agarwood

Agarwood refers to aromatic oleoresin resulting from pathological activity in the natural wood of Thymeleacea and Gyrinops. When

heated, this oleoresin has unique fragrance properties, making it in demand in the perfumery industry (Ismail et al., 2016; Jayachandran et al., 2014). Resinous agarwood emits a unique soft fragrance when burnt, but its scent is easily detected in super quality agarwood. Distributed in different countries, agarwood is called with a different language and usages relevant to local culture. In Malaysia and Indonesia, agarwood is known as 'gaharu' or 'karas' (Chua, 2008). For the Arabic community who consumes agarwood as raw material in perfumery, they name gaharu as 'oud' (Jung, 2013). In China, agarwood is familiar as 'chen xiang', while in Hindi as 'agar' (Akter et al., 2013). In Vietnam, native people named agarwood 'tram huóng' (Nguyen, 2014). In Japan, agarwood is known as jinkō (Jung, 2013). While vernacular name sound confusing sometimes, their commercial name is widely known as agarwood, aloeswood and eaglewood (Chua, 2008). Quality of agarwood is related to plant species, human engagement, microorganism interaction and some other abiotic factors.

3.1. Agarwood formation

A healthy tree of *Aquilaria* spp. will never generate resin compound. It has to be exposed to certain stimulants to stimulate plant defense mechanisms to act, hence, allowing the production of volatile compounds and aromatic secretion of resin. As demonstrated by Rahayu (2010), healthy wood transforms into agarwood after artificial induction. It is also found that sugar content in agarwood inoculant will help agarwood tree defense mechanism by restraining clump formation symptoms. *A. sinensis* converts substances found in the healthy tree into agarwood compound at eight months of incubation. Some available chemical substances in a healthy tree, such as dibutyl phthalate and phenol,2,2-methylenebis[6-11-dimethylethyl)-4-methyl] are decreased after fungi invasion (Cui et al., 2013). This discovery is supported by researchers (Chen et al., 2011) when different chemical compounds were identified in healthy trees, chemical-inoculated trees and natural agarwood. Substance conversion is initiated by signal molecules produced by fungi during infection. Thus, it stimulates gene expression linked to the synthesis of secondary metabolites and eventually their accumulation. However, compound concentration varies based on cellulose and lignin from the stem part (Novriyanti and Santosa, 2011). The first process of olio-resin production is taken in cells of the included phloem, rays, parenchyma para-trachea, and trachea. It is the tree's response to injury if its first line of defense, the formation of phloem callus tissue, is inhibited from forming over the wound (Gunn et al., 2004; Mulyaningsih, 2002). As shown in Figure 1, the agarwood compound will be produced after interaction with fungi (*Fusarium* sp.) and insects. Compounds found in agarwood were synthesized through lipogenase (LOX) and mevalonate pathway (MVA). The concerned pathway is the MVA pathway that converts acetyl Co-A to terpenoid, significantly essential to contributing to agarwood's unique fragrance.

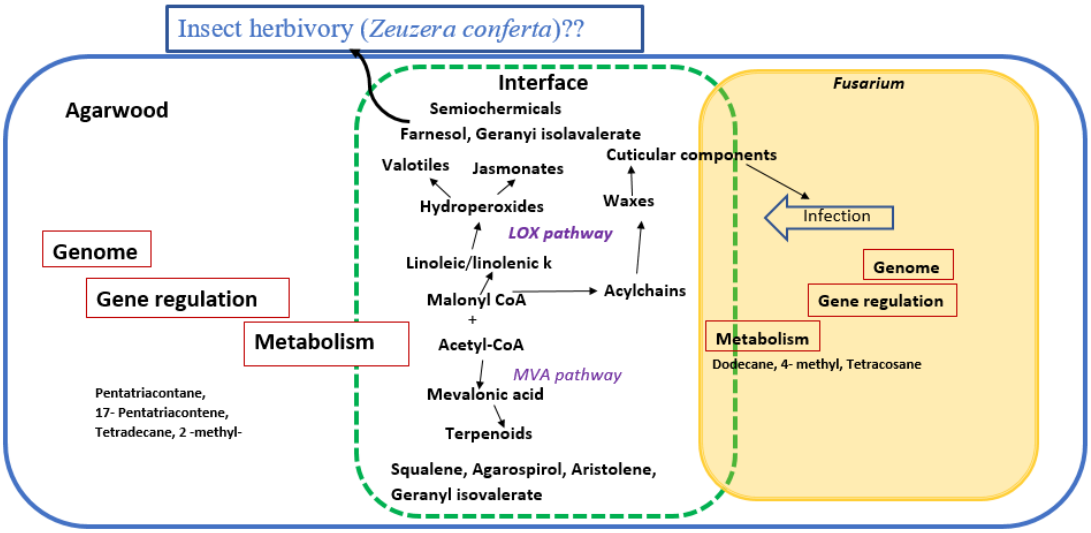

Figure 1. Interaction of fungi (*Fusarium* sp.) and insect with agarwood healthy wood lead to the production of biochemicals associated with agarwood (Pinney, 2011)

3.2. Plant defense mechanism

The plant defense mechanism will induce a secondary plant metabolite known as sesquiterpene. Sesquiterpene is one of the volatile plant compounds that can be antibacterial, antifungal or repellent to herbivores (Huang et al., 2012; Taniguchi et al., 2014). Sesquiterpenes are a terpene class consisting of three isoprene units and have the molecular formula $C_{15}H_{24}$. Sesquiterpenes may be acyclic or contain rings, including many unique combinations. Biochemical modifications such as oxidation or rearrangement produce the related sesquiterpenoids. Sesquiterpenes are found naturally in plants and insects (Zviely and Li, 2013). The formation of sesquiterpenes as a result of plant interruption was proven in the plant family solanacea (Santoso et al., 2011).

However, compound formation and accumulation of agarwood have critically relied on types of stimulants. Yet, specific responses mechanism before agarwood formation remain a mystery, where only seven percent of total agarwood from nature will be infected and produce agarwood (Akter et al., 2013). The healthy tree may respond differently to microbial infection that is eventually associated with agarwood formation through the level of disease and synthesizing chemical compounds (Santoso et al., 2011). Decomposed part of the trunk contributes to the scented properties of agarwood (Novriyanti and Santosa, 2011). When healthy trees are affected by fungi invasion and injury, plant cells begin to strengthen physical protection by cell thickening and lignification (Cui et al., 2013). As infection occurs at the injured part, tylosis may occur to cut off nutritional supply from being used by the pathogen (Akter et al., 2013). Tylosis is an abnormal growth of parenchyma cells in

xylem vessels occurring in secondary heartwood when the tree is stressed or distraught. Apart from that, plant self-injury or self-death system is necessary to block fungal spread by stopping food supply and further infection areas with lignification (Bednarek, 2012; Cui et al., 2013). The plant defense system is part of surrounding management security (Bednarek, 2012). The alarm system will be triggered when the microbe-associated molecular patterns (MAMPs) receptor identifies a threat. Secondary metabolites are usually produced with antimicrobial and toxicity properties to stop the infection from spreading to other healthy plant cells. For instance, benzoxazinone glucosides (BXs) may be indicated by the disorganization of plant cell structure (Frey et al., 2009). Therefore, interruption in plant security may trigger the plant defense system to initiate the formation of secondary metabolites to rescue healthy cells. In agarwood, the secondary metabolite accumulation appears physically in the form of aromatic resin substance.

3.3. Fungi association in agarwood formation

In nature, agarwood formation comes from responses to fungal infection and physical wounding (Barden et al., 2000). Previous studies reported that more than one fungus species interact with *Aquilaria* sp. during agarwood formation and that one tree may have at least three fungi association (Mohamed et al., 2014; Nurbaya and Baharuddin, 2014; Rahayu, 2010; Santoso et al., 2011). For example, Santoso et al. (2011) thriven to isolate *F. sambunicum*, *F. tricinctum*, and *F. solani*, the most dominant species in agarwood samples from several locations in Indonesia. Involvement of *Fusarium* spp. is strongly proven by the growth of nine isolates on Potato Dextrose Agar (PDA) and Banana Leaf Agar (BLA) consisting of *F. solani*, *F. oxysporum*, *F. lateritium*, *F. comfactum*, and *Fusarium* spp. based on fungal morphology (Nurbaya and Baharuddin, 2014).

F. solani is a common pathogenic fungus and decomposer in Malaysia that exists in roots, stems, trees, and soil (Chehri et al., 2015; Shahnazi et al., 2012), and it also can be found in all agarwood samples. Characterization of *Fusarium* sp. was referred to Leslie and Summerell (2006), Hafizi et al. (2013), and Chehri et al. (2015). Generally, microconidia were mostly oval shape and non-septate, with occasional septate and elongated forms. Meanwhile, macroconidia will be produced at the inoculation point known as sporodochia. Macroconidia characteristics could be summarized as usually straight, some were slightly curved, 4-5 septa with mostly 5 septa, blunt, apical cell shape, and a barely notched basal cell.

Besides *Fusarium* species, new findings indicate that *Xylaria* spp. and *Lasiodiplodia* spp. could be involved in agarwood formation (Cui et al., 2013; Mohamed et al., 2010). *Mucor* sp. from the white wood sample, known as T3, turned out to be *Cunninghamella bainieiri* after a DNA sequence analysis based on the NCBI database. In contrast, unknown species from T8 and T9 resinous wood samples have been identified as *Lasiodiplodia theobromae* (Mohamed et al., 2010). Identification of *L. theobromae* was based on colony and conidia, which takes about four weeks to produce (Norhayati et al.,2016). *L. theobromae* found in Peninsular Malaysia was determined to be pathogenic against *Jatropha curcas*, *Acacia mangium*, and *A. crassicarpa* species (Sulaiman et al., 2012; Tarigan et al., 2011).

Other fungi found in the same research were *Curvularia* sp. and *Trichoderma* sp. All fungi identification was done based on a molecular approach without fungi plate culture. However, different fungi species were also discovered using molecular, and fungi culture approaches. Agar-plating technique revealed *Trichoderma* sp., *Alternaria* sp., *Fusarium* sp., *Curvularia* sp., *Cladosporium* sp., and *Phaeoacremonium* sp. from matured agarwood of *A. malacensis* in India (Premalatha and Kalra, 2013). These fungi were confirmed physically and genetically. In addition, although fungi *Preussia* sp. and *Phaeoacremium africana* could not be physically identified in plate culture, they were discovered after molecular analysis.

The interaction between agarwood and fungi leaves was found based on the biological activity changes in agarwood trees. Enzymes like cellulose, pectinase, peroxidase, and polyphenol oxidase showed higher activity in *A. malaccensis* infected with *Chaetomiun globosum* and *F. oxysporum* (Tamuli et al., 2011). Meanwhile, *Aspergillus* spp. isolates AR13 affect higher laccase, cellulase and ligninolytic enzyme in agarwood tree while breaking down tree carbohydrate in comparison with *Penicilium* sp., *Fusarium* sp., *Chaetomium* sp., and *Lasiodiplodia* sp. (Sangareswari et al., 2016). *Aspergillus* genus isolated from *A. sinensis* has been recorded in China (Cui et al., 2013) and in *A. malaccensis* in India (Premalatha and Kalra, 2013). In Peninsular Malaysia, *Aspergillus* spp. was widely found infecting food and soil and decomposed rubber tree and oil palm trees (Lee, 2012). Little has been discussed if *Aspergillus* spp. may exist as endophytes in agarwood trees. *Aspergillus* spp was reported as an endophyte in the plant *Taxus mairei*, (Zhang et al., 2008), soybean plant (Khan et al., 2011), and *Melia azedarach* (Li et al., 2012). Known as common soil-born fungi, *Aspergillus* spp. may interact with plants as a pathogen and endophyte.

The effect of fungi roles in contributing to the formation of the unique aroma of agarwood has become clearer in recent research. Accumulation of agarwood compounds infected with *Fusarium* showed the most variation in chemical compound types (44 compounds) compared to uninjured control and injured control of juvenile agarwood trees in Assam after three months-induction (Sen et al., 2017). Among 44 compounds, 11 (eleven) compounds were discovered in agarwood samples either as oil or chip wood which were different from previous research.

3.4. Artificial agarwood formation in plantation

Previous research discovered a fungal association with natural agarwood. Thus, the initiative to produce artificial agarwood started with the selection of fungi. More than one single species was found from the fungal screening of one agarwood sample (Cui et al., 2013; Mohamed et al., 2010; Premalatha and Kalra, 2013). Hence, resin accumulation in agarwood may be induced by the combination of fungal species action. Despite the findings, incorrect fungi choice and the combination will only bring perish to plant and agarwood quality. Inappropriate fungal induction may disrupt plant cells and degrade resin that has formed and left rotted (Putri et al., 2017). Although some fungal inoculant may affect negatively, effective fungal inoculant also shows physical changes to the part of the agarwood tree other than the stem. According to Rahayu (2010), in artificial inoculation using potential fungi species, chlorosis symptoms appear on leaves on the first and second branches closest to injected holes after a month of injection. When the frequency of inoculants increases, the symptom will be diverse in other parts of the plant. The second month after artificial inoculation, injection holes start to darken and their intensity increase.

The fungal effect varies depending on the duration of infection. It is claimed that the longer the infection occurs, the intensity of colour and disease area will increase, respectively. The area of infection will turn brownish or darker colour. Darker colour with the larger area of the infected area from the inoculated point was observed on six

months of induced agarwood compared to three months of induced agarwood (Mohamed et al., 2014). The dark colour on the wood indicated agarwood formation where the resin was secreted during interaction with fungal inoculation. The intensity of colour suggested an abundance of various types of aromatic compounds. As in *A. sinensis*, it takes at least eight months of fungal inoculation before it can be transformed into an agarwood compound (Cui et al., 2013). While resin accumulation intensifies, the fungal community is decreasing slowly. Reviewing the agarwood formation pattern after inoculating with *F. solani*, *Cunninghamella bainieri*, and *L. theobromae*, the population of *F. solani* and *C. bainieri* declined except for *L. theobromae* as quantified using qPCR (Mohamed et al., 2014). The findings may be caused by the properties of resin in agarwood and the plant's action to defense its healthy cells. Mature agarwood has aromatic compounds such as anisylacetone, benzaldehyde, benzylacetone and some sesquiterpene groups; these compounds contribute to fragrance properties, and they also proved to be anti-microbial (Chen et al., 2011; Hendra et al., 2016).

4. Fungi as agarwood inoculant

Artificial agarwood is produced by injecting agarwood inoculant into the matured agarwood tree. Inoculant is believed to contain agarwood inducer factors, including fungi. According to Mohamed et al. (2010) and Rahayu (2010), inoculation using a combination of *Acremonium* spp. and *Fusarium* spp. is more reliable to induce the production of aromatic compound accumulation than single species. Better wood discolouration, length, the width of the infected area, and fragrance was determined with *Acremonium* spp. inoculation followed by *Fusarium* spp. inoculation after one week. Unfortunately, no agarwood chemical analysis was done to verify its chemical compound content. *Fusarium* spp. is the most favorable fungal candidate to be included in agarwood inoculant. However, the region of origin may differentiate the potential of *Fusarium* spp. as the best inducer. It was found that *Fusarium* spp. originating from Tamiang Layang, Indonesia, has the highest confirmed composition of agarwood. Meanwhile, *Fusarium* spp. from Maluku belongs to the highest total concentration of aromatic compounds found in artificial agarwood of *A. microcarpa* (Novriyanti and Santosa, 2011). The discovery was revisited when three *Fusarium* spp. as *F. solani*, *F. sambunicum*, and *F. tricintum* isolated from agarwood in Gorontolo area, Indonesia, proved to be the most effective agarwood inducer in *A. malaccensis* and *A. microcarpa* (Santoso et al., 2011). The fungi can induce agarwood within two to six months after inoculation. Inoculation of *A. sinensis* in China using various fungi such as *Pestalotiopsis* sp., *Xylaria* sp., *Fusarium* sp., *Trichoderma* sp, *Colletotrichum gloeosporioides*, *Xylaria* sp., *Chaetomium* sp., *Penicillium* sp., and *B. rhodina* and *B. rhodina* combined with formic acid induction produced high yield and high quality of agarwood after seven to 12 months (Tian et al., 2013).

Recent agarwood inoculation research finds that fungal inoculant is the best to induce sestquiterpene production. Inoculant composed of fungi *Phialophora* sp. and *Fusarium* spp. successfully generated 42.2 % sesquiterpene content, the highest value compared to control and chemically induced agarwood of *A. crassna*. The result was derived from two years inoculation period. Apart from fungi, some agarwood producer applies salt and chemical such as methyl jasmonate and hydrogen peroxide to agarwood tree to induce resinous wood (Kenmotsu et al., 2011; Zhang et al., 2014). Some non-biological inducers may have induced agarwood, while others tend to kill the tree.

5. Agarwood quality

In the agarwood market, there are different prices of agarwood based on its quality. The traditional measurement of agarwood quality is reliable among agarwood traders since no standard agarwood quality has been established until now. However, agarwood price classification is not rigid and subjective according to individual or culture. Meanwhile, current technology application is more rigid and accurate where compound detection and amount are specific, and the result is reproducible.

5.1. Agarwood chemical compounds

Fragrance in agarwood comes from volatile compounds in the terpene group. Terpenoid metabolism may cause the metabolic pathway past sesquiterpenoid production when harvested (Rahayu, 2010). Natural agarwood gets higher demand in the international market because natural agarwood gives a softer, unique scent than artificially inoculated agarwood. The chemical constituent is made differently based on artificial induction, natural induction and undisturbed healthy tree. Chen et al. (2011) found that a healthy tree has the highest total volatile at 95.68%, followed by natural agarwood at 92.16% and chemical induction at 90.01 %. However, as defined by aromatic wood, chemical induction agarwood and natural agarwood comprises sesquiterpenes and aromatics of 80.00% and 89.01% compared with a healthy tree with a significant fatty acid and alkanes. Some terpene sub-groups such as sesquiterpenes and aromatics found in chemical induction and natural agarwood are benzylacetone, guaia-1(10),11-dien-9-one, guaiol, hinesol, α-copaen-11-ol, baimuxinal, vanillin, α-humulene, α-agarofuran, elemol, agarospirol, aromadendrene oxide, and baimuxinal. Natural agarwood has the least composition of alkanes and fatty acids, which indicates the superior quality of its agarwood.

5.2. Production of terpenes

Any stress of threat to plant biological activity may induce the production of secondary metabolites, which allows the plant to adapt and survive. For instance, a rubber tree will produce latex when its stem is cut open, which means recovery. According to Angelova et al. (2006), the substance is released as an act of defense in the plant. Secretion of phytoalexin, latex, and terpenoid is proof of plant self-healing, counterattack to pathogens and self-protection against environmental pressure (Frost et al., 2008; Maffei, 2010). Plant are able to release volatile compounds as a mean to communicate to adjacent plants, to attract a predator to get on plant biological threats or as an act of defense (Degenhardt, 2009).

Terpene is one example of a volatile compound and plant secondary metabolite with more than 30.000 groups (Parveen et al., 2014). It is biosynthesized through the mevalonate pathway and released by abiotic factors, especially from temperature fluctuation by *Citrus* (Wang et al., 2012). Terpene production happens in cytosol and plastid, where cytosol operates the MVA pathway while plastid has 1-deoxy-*d*-xylulose 5-phosphate (DXP) pathway, as shown in Figure 2. Unlike abiotic factors, biotic factors such as microbes, fungi, and herbivores get in physical contact with targeted plant organs and can also stimulate terpene biosynthesis. As in *A. malaccensis*, any injuries and pathogen contact will stimulate the production of resin consisting of terpene. These have also been demonstrated by the production of azadirachtin-A, a type of tetranortriterpenoid, during infection of *Agrobacterium rhizogenes* with *Azadiracta indica* (Satdive et al., 2007).

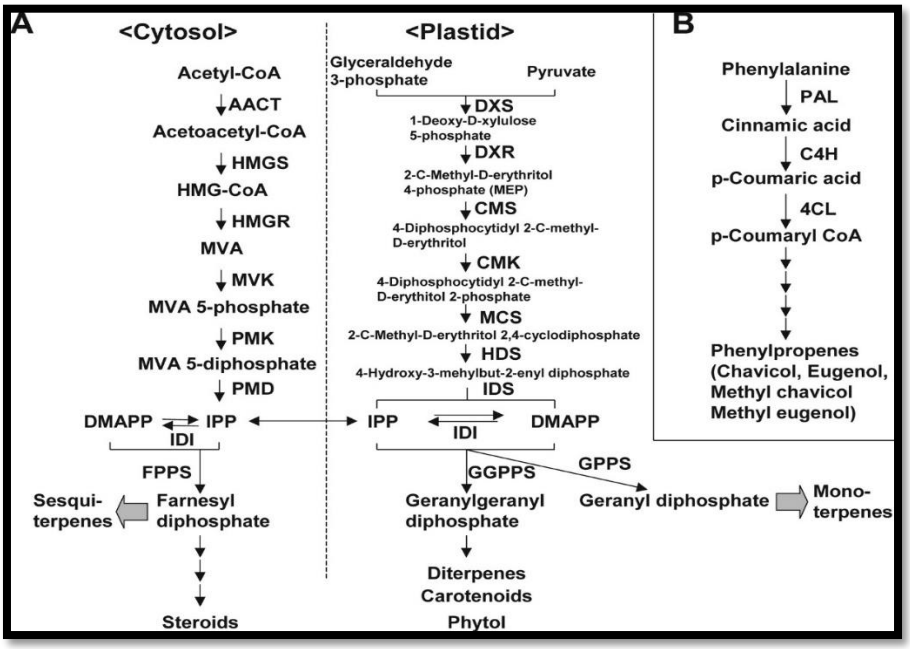

Figure 2. Terpene production happens in cytosol and plastid where cytosol operates MVA pathway while plastid has 1-deoxy-*d*-xylulose 5-phosphate (DXP) pathway.
MVA generally produces sesquiterpene from farnesyl diphosphate and geryal diphosphate in DXP pathway.

6. Agarwood quality determination

Agarwood quality determination based on the traditional method is more associated with human scent and perception like aroma and country of origin. Agarwood physical properties such as density, colour, and scent are also applied in the agarwood grading system.

6.1. Human scent and perception

Individual enjoyment of different kinds of smell for leisure is different; some are already bonded with cultural practice. Agarwood may emit a sweet, woody and spicy aroma. In the agarwood direct selling market, the scent was the primary consideration for preference (Antonopoulou et al., 2010). Arabic people prefer woody and spicy aromas, while Chinese and Japanese inclined to choose sweet and fruity smells (Liu et al., 2017). These aromas may be significant to their country of origin resin content of the agarwood and *Aquilaria* species. However, agarwood with low quality will release an irritating burning aroma to the eyes and nose when burnt. A pleasant smell will only come from high-quality agarwood and the scent may remain in a room for a longer period depending on its resin and agarwood size (Antonopoulou et al., 2010; Liu et al., 2017). After all, agarwood quality based on human perception is unreliable as an indicator determining agarwood price in the general world market.

6.2. Agarwood physical properties

Resin content in agarwood may differentiate high-quality agarwood from low-quality agarwood based on colour density and sinkage.

6.2.1. Agarwood color

Resin accumulation after the agarwood tree is infected may affect the colour density of agarwood: the darker agarwood, the higher quality of agarwood (Barden et al., 2000). In the UAE agarwood market, sellers purposely polished agarwood to make it look darker than the original colour to attract buyers (Antonopoulou et al., 2010). Another trick was that sellers acquired immature agarwood and buried it in the soil for a certain time to make it look darker after prolonged decomposition (Heuveling van Beek and Phillips, 1999). Therefore, agarwood color was a disputable judgement for agarwood quality. Nevertheless, Malaysia agarwood still applies agarwood quality based on agarwood darkness named 'ABC' grading system. Grade A agarwood called 'kalambak' is dark brownish wood, Grade B is healthy wood with dense strips of brownish resin, while Grade C is a lesser brownish resin strip compared to Grade B (Liu et al., 2017).

6.2.2. Agarwood sinkage

Higher quality agarwood is considered to have entire sinkage property as it contains abundant resin. This method is to put agarwood in water and measure its position, either sinkage, half sinkage or floating. Sinkage agarwood indicates high-quality agarwood, half-sinkage to become medium-quality agarwood and finally, floating agarwood is considered low-quality agarwood (Liu et al., 2017). The sinkage justification was an inaccurate measurement, as not all high-quality agarwood will sink. The sinkage phenomenon is associated with wood density which accounts for types of *Aquilaria* spp. For instance, a lower grade of *A. malaccensis* can sink more profoundly than the high quality of *A. sinensis* since *A. malaccensis* has a compact texture and denser wood than *A. sinensis* (Liu et al., 2017).

6.2.3. Chemical analysis of agarwood

The quality of agarwood can be analyzed from agarwood oil, agarwood smoke or agarwood volatile compound. Compounds from chip wood samples were extracted using the Solid Phase Microextraction (SPME) method. This method allowed samples free from any alteration or disturbance by solvent where all volatile

compounds evaporate directly from agarwood and adhere to SPME fiber. The method introduced by Arthur and Pawliszyn (1990) was known to be a rapid, sensitive and solvent-free method. Moreover, the SPME method is fast and straightforward in analyzing agarwood quality compared to any other extraction SPME method that requires fiber to trap volatile compounds (Meng et al., 2011; Pripdeevech et al., 2011). According to a study of volatile compounds from virgin coconut oil, divinylbenzene-carboxy polydimethylsiloxane (DVB-CAR-PDMS) was found to be the most suitable due to its high sensitivity to exposed standard combination with a relative standard deviation that is less than 10% of concentration (1.0 µg/g) compared to carboxen polydimethylsiloxane (CAR-PDMS), polydimethylsiloxane-divinylbenzene (PDMS-DVB) and polydimethylsiloxane (PDMS) coating fibers (Vichi et al., 2003). In addition, DVB-CAR-PDMS was firmly proven to be efficiently applied with a broader range of sample concentrations up to 5 µg/g and gave consistent results (Vichi et al., 2003).

GC-MS is a reliable instrument to detect compound composition in volatile substances based on compound mass. Previously, aromatic samples were qualified based on odour sensed by a human. However, it is subjective to rely on. On the other hand, GC-MS measures compound compositions based on gas chromatography and mass spectrometry. Gas chromatography uses a capillary column, which may vary with length, diameter, film thickening, and phase property. The molecules will be separated in the capillary column during GC-MS operation based on their molecular mass. Therefore, each unique compound will reach the end of the capillary column at different times due to its chemical properties. Compounds that move out of the capillary column will be ionized and detected based on the mass-to-charge ratio with a mass spectrometer (Hübschmann, 2015). GC-MS has been widely used to identify compounds in essential oil. GC-FID and GC-MS analysis helped discover of fundamental oil sesquiterpene group (Maggi et al., 2010). Previously, GC-MS application identified the main compounds of *Thymus corosus* Heuff or thyme named cryophyllene-oxide, camphene, β-bourbonene, eudesmol, and α-pinene (Pavel et al., 2009). As essential oil consists of monoterpene and sesquiterpene, GC-MS successfully differentiated *Tangetes minuta* L. or chinchilla elemental oil composition among different origins (Chamorro et al., 2012). These repeated operations of GC-MS also lead to agarwood composition analysis from other species, locations and maturity (Chen et al., 2011; Tsan and Mohamed, 2014). While GC-MS alone may not be robust data, GC-MS and GC-FID combination will give rigid data. GC-FID analysis is based on a flame ionization detector and is practically applied to organic compounds (Holm, 1999). GC-FID reflects unknown compounds according to their Kovats Index, calculated with known carbon standard retention time when GC-MS is limited with the available database. A combination of GC-MS and GC-FID has been practised with essential oil composition of *Coriandrum sativum* L, saffron and beer volatile analysis (Anastasaki et al., 2009; Charry-Parra et al., 2011; Msaada et al., 2007).

7. Commercial value of agarwood

High demand in the perfumery industry has led to illegal trade and threats to the agarwood tree population in its natural habitat. Natural agarwood reaches international prices from USD 2.000 to USD 16.000 per kilogram (PERsOOn, 2007). Agarwood planters generate luxurious income for their consistent high price. Agarwood is being exported in the form of chips, powder and oil. Price is dependent on resin content and purity of agarwood. Agarwood chips may be sold from USD 29.0 to USD 8888.0 per kilogram. High-quality agarwood starts at USD 1.000/kg (PERsOOn, 2007). For every initial investment of USD 32.000, agarwood traders can gain a return of USD 104.000. Hence, the trader made up a 29% Internal Rate of Return within seven years of trading (Akter et al., 2013). Meanwhile, agarwood essential oil costs between USD 50.000 to USD 80.000 per litre, requiring extraction of 100 to 150 kg of agarwood (PERsOOn, 2007).

International export value is estimated at USD 14 million, which is 43% of the total retail value. Laos produces agarwood supply to the world worth USD 33 million, where the majority is contributed by a foreign harvester who exports about 18 tonnes of agarwood to Vietnam (Jensen, 2009). If the East-Asian market is considered, estimates of overall retail value from Laos would be higher, USD 40 to 45 million. In 2004, international trade in agarwood was assumed to reach USD 2.3 billion (Jensen, 2009). Mai dii, a common name for high-grade agarwood in Laos, costs more than THB 500/kg for raw agarwood. Medium grade, known as mai khilai, goes for extraction of top-quality essential oil and is marketed at THB 80 to 500 per kg (Jensen and Meilby, 2010). These harvesters collect profit when they can better network with big agarwood industry players. They sell agarwood at higher prices when agarwood industry players gain the advantage of value-added agarwood products and earn more than harvesters (Jensen and Meilby, 2010).

As a consequence of this scenario, agarwood industry players are torn between limited high-quality resources and high prices for business sustainability. Another alternative to agarwood is derived from a chemical process despite the highly preferred natural agarwood (Akter et al., 2013). Japan maintains its demand for the rare top quality natural agarwood (PERsOOn, 2007) and the United Arabic Emirates is included in using natural agarwood in religious rituals. Excellence in commercial markets and trade is the leading cause of *Aquilaria* sp. extinction.

Overharvesting, uncontrollable illegal logging and reserved forest invasion by local or neighbouring citizens bring *Aquilaria* sp. loss and habitat to endemic flora and fauna (Akter et al., 2013). *A. malaccensis* is in the list of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Inskipp and Gillett, 2005). Therefore, rather than hunting agarwood in their natural habitat, conducive climate countries have initiated their agarwood plantation as in Bangladesh, Bhutan, India, Indonesia, Laos, Malaysia, Myanmar, Vietnam, Australia, and Thailand.

8. Application of agarwood

Nowadays, agarwood is in top demand in the perfumery industry. Nevertheless, the multipurpose of agarwood has made it a demanded forest source for decades. Incorporating agarwood aroma into human life and discovering traditional agarwood practices open a new field for researchers to explore the further discovery of this agarwood potential. Continuous agarwood practice in human lifestyle and lengthy application in health improvement bring agarwood to become a world brand, although its supply is depleting each year.

8.1. Agarwood in culture and ritual

Since ancient times when our ancestors accidentally found agarwood in the forest, they brought agarwood into usefulness and adapted it to the local culture. For the Malay race, it is widely used in the range of perfume, religious purposes, spiritual rituals, and decorative carvings (Chua, 2008; Saikia and Khan, 2012). Even today, some agarwood field practitioners made low-quality agarwood into

attractive shapes and bracelets for sale. For Muslims, non-alcoholic perfume is preferred during prayer because agarwood-originated perfume has become popular among Arabic people. Besides, the simplest way to enjoy agarwood fragrance is by burning, happily applied to the luxury incense-making industry. Agarwood incense would not contain total agarwood composition due to its limited supply and high price. Nonetheless, agarwood incense is used in the religious ritual activities of Muslims, Buddhists and Hindus (Akter et al., 2013).

8.2. Agarwood in health and medicinal purpose

Apart from being an aromatherapy material, in medicinal prospect, agarwood can be used as a carminative, stimulant for heart palpitation, tonic during pregnancy, remedy during the post-natal recovery period, and cure for the disease of the female genital part. The whitebark of agarwood is believed to heal jaundice and body pain (Ali et al., 2016). Moreover, agarwood helps to relieve body pain, warm abdomen, relieve asthma, treat coughs, and acroparalysis, and acts as an anti-histamine, analgesic, and anti-inflammatory (Bhuiyan et al., 2009; Chitre et al., 2007). In Chinese Traditional Medicine, it is helpful as a sedative to relieve gastric problems, relieve rheumatism and high fever (Liu et al., 2017). Various compounds in agarwood can fight cancer cells but the information is limited (Dahham et al., 2014). Sesquiterpene compounds in agarwood pose antimicrobial properties and they are effective, especially to gram-positive bacteria (Chen et al., 2011).

8.3. Agarwood as perfume

Practice commonly among Arabic culture and agarwood lovers has spread worldwide as a component in fragrant commercial brands. In Yemen culture, agarwood aromatic is indulged in several ways (Jung, 2011). The smoking odour of raw agarwood chip wood is applied during prayer, special occasions and daily home refresher. Usually, dense, resinous agarwood turns into incense compared to low-quality agarwood. Lower-grade wood will be distilled to extract pure agarwood oil. Some people, mainly Arabic, enjoy the original woody, balsamic scent of agarwood oil. However, agarwood oil may undergo adulteration to satisfy a wider range of people's preferences in fragrance (Jung, 2013). It can be blended with other fragrances as base notes to lengthen the fragrance effect. Another way to add value to lower-grade agarwood is to immerse low-grade chip wood in agarwood oil before being sold as incense, prayer bead, sculpture and home decoration (Jung, 2011). Agarwood oil from different species and regions will emit different scents due to their difference in the top note, such as fruity, animal and leathery odour (Zviely and Li, 2013). Notes depend on the volatility of compounds in particular fragrances. Top notes have the highest volatility, followed by medium and base notes. In the agarwood case, regardless of species and region, the oil will definitely emit a woody smell after seven to eight hours of skin application. These woody smells may be contributed by compounds such as eremophila-9,11-dien-8-one, (-)–guaia-1(10), 11-dien-15-al, and jinkohol II (Zviely and Li, 2013).

9. Conclusions

Plant compounds derived from agarwood have long been utilized as traditional remedies in Southeast Asian societies and Chinese, Tibetan, Unani, and Ayurvedic medicine. They have sedative properties and are used to treat arthritis, asthma, and diarrhoea. Natural agarwood induction periods were unknown but considered acceptable for harvesting and marketing by Kedaik agarwood practitioners. Agarospirol, β-selinene and 10-epi-γ-eudesmol were detected in artificial and high-quality natural agarwood, where agarospirol existed in higher abundance in artificial agarwood. Many pharmacological studies on crude extracts have been conducted. These extracts exhibit anti-allergic, anti-inflammatory, anti-diabetic, anti-cancer, antioxidant, anti-ischemic, antimicrobial, hepatoprotective, laxative, and mosquitocidal properties and central nervous system effects. Future research should focus on bioassay-guided isolation of bioactive molecules with adequate chemical characterization, as well as insights into the underlying processes for drug development. By tying agarwood ethnopharmacology to reported pharmacological qualities, anti-inflammatory capabilities may be the future study area, as inflammation underpins many disease states.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Statement of ethics

In this study, no method requiring the permission of the “Ethics Committee” was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary File

None.

References

Akter, S., Islam, M.T., Zulkefeli, M., Khan, S.I., 2013. Agarwood production-a multidisciplinary field to be explored in Bangladesh. *International Journal of Pharmaceutical and Life Sciences*, 2(1), 22-32.

Ali, N.A.M., Jin, C.B., Jamil, M., 2016. Agarwood (*Aquilaria malaccensis*) Oils Essential Oils in Food Preservation, *Flavor and Safety* (pp.173-180): Elsevier.

Anastasaki, E., Kanakis, C., Pappas, C., Maggi, L., Del Campo, C.P., Carmona, M., Polissiou, M.G., 2009. Geographical differentiation of saffron by GC–MS/FID and chemometrics. *European Food Research and Technology*, 229(6), 899-905.

Angelova, Z., Georgiev, S., Roos, W., 2006. Elicitation of plants. *Biotechnology & Biotechnological Equipment*, 20(2), 72-83.

Antonopoulou, M., Compton, J., Perry, L.S., Al-Mubarak, R., 2010. The trade and use of agarwood (Oudh) in the United Arab Emirates. TRAFFIC Southeast Asia, Petaling Jaya, Selangor, Malaysia.

Arthur, C.L., Pawliszyn, J., 1990. Solid phase microextraction with thermal desorption using fused silica optical fibers. *Analytical Chemistry*, 62(19), 2145-2148.

Barden, A., Anak, N.A., Mulliken, T., Song, M., 2000. Heart of the matter: agarwood use and trade and CITES implementation for *Aquilaria malaccensis*. TRAFFIC International, Cambridge, UK.

Bednarek, P., 2012. Chemical warfare or modulators of defence responses–the function of secondary metabolites in plant immunity. *Current Opinion in Plant Biology*, 15(4), 407-414.

Bhuiyan, M.N.I., Begum, J., Bhuiyan, M.N.H., 2009. Analysis of essential oil of eaglewood tree (*Aquilaria agallocha* Roxb.) by gas chromatography mass spectrometry. *Bangladesh Journal of Pharmacology*, 4(1), 24-28.

Chakrabarti, K., Kumar, A., Menon, V., 1994. Trade in agarwood: TRAFFIC-India, WWF, India.

Chamorro, E.R., Zambón, S.N., Morales, W.G., Sequeira, A.F., Velasco, G.A., 2012. Study of the chemical composition of essential oils by gas chromatography. *Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications*, 1, 307-324.

Charry-Parra, G., DeJesus-Echevarria, M., Perez, F.J., 2011. Beer volatile analysis: optimization of HS/SPME coupled to GC/MS/FID. *Journal of Food Science*, 76(2), C205-C211.

Chehri, K., Salleh, B., Zakaria, L., 2015. Morphological and phylogenetic analysis of *Fusarium solani* species complex in Malaysia. *Microbial Ecology*, 69(3), 457-471.

Chen, H., Yang, Y., Xue, J., Wei, J., Zhang, Z., Chen, H., 2011. Comparison of compositions and antimicrobial activities of essential oils from chemically stimulated agarwood, wild agarwood and healthy *Aquilaria sinensis* (Lour.) Gilg trees. *Molecules*, 16(6), 4884-4896.

Chitre, T., Bhutada, P., Nandakumar, K., Somani, R., Miniyar, P., Mundhada, Y., Jain, K., 2007. Analgesic and anti-inflammatory activity of heartwood of *Aquilaria agallocha* in laboratory animals. *Pharmacol Online*, 1, 288-298.

Chua, L.S.L., 2008. Agarwood (*Aquilaria malaccensis*) in Malaysia. Forest Research Institute Malaysia.

Cui, J., Wang, C., Guo, S., Yang, L., Xiao, P., Wang, M., 2013. Evaluation of fungus-induced agilawood from *Aquilaria sinensis* in China. *Symbiosis*, 60(1), 37-44.

Dahham, S.S., Ahamed, M.B.K., Saghir, S.M., Alsuede, F.S., Iqbal, M.A., Majid, A.M.S.A., 2014. Bioactive essential oils from *Aquilaria crassna* for cancer prevention and treatment. *Global Journal on Advances Pure and Applied Sciences*, 4, 26-31.

Degenhardt, J., 2009. Indirect defense responses to herbivory in grasses. *Plant Physiology*, 149(1), 96-102.

Duriyaprapan, S., Khuankhamnuan, C., Tanpanich, S., 2003. The plant resources of south-east Asia (PROSEA). In III WOCMAP Congress on Medicinal and Aromatic Plants-Volume 1: Bioprospecting and Ethnopharmacology 675 (pp. 15-21).

Elias, M.F., Ibrahim, H., Mahamod, W.R.W., 2017. A review on the malaysian *Aquilaria* species in karas plantation and agarwood production. *International Journal of Academic Research in Business and Social Sciences*, 7(4), 1021-1029.

Frey, M., Schullehner, K., Dick, R., Fiesselmann, A., Gierl, A., 2009. Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. *Phytochemistry*, 70(15-16), 1645-1651.

Frost, C.J., Mescher, M.C., Carlson, J.E., De Moraes, C.M., 2008. Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiology*, 146(3), 818-824.

Gunn, B., Stevens, P., Singadan, M., Sunari, L., Chatterton, P., 2004. Eaglewood in Papua New Guinea.

Hafizi, R., Salleh, B., Latiffah, Z., 2013. Morphological and molecular characterization of *Fusarium solani* and *F. oxysporum* associated with crown disease of oil palm. *Brazilian Journal of Microbiology*, 44, 959-968.

Hendra, H., Moeljopawiro, S., Nuringtyas, T.R., 2016. Antioxidant and antibacterial activities of agarwood (*Aquilaria malaccensis* Lamk.) leaves. In AIP Conference Proceedings (Vol. 1755, No. 1, p. 140004). AIP Publishing LLC.

Heuveling van Beek, H., Phillips, D., 1999. Agarwood: trade and CITES implementation in Southeast Asia. Unpublished report prepared for TRAFFIC Southeast Asia, Malaysia.

Holm, T., 1999. Aspects of the mechanism of the flame ionization detector. *Journal of Chromatography A*, 842(1-2), 221-227.

Huang, M., Sanchez-Moreiras, A.M., Abel, C., Sohrabi, R., Lee, S., Gershenzon, J., Tholl, D., 2012. The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene *(E)*-β-caryophyllene, is a defense against a bacterial pathogen. *New Phytologist*, 193(4), 997-1008.

Hübschmann, H.J., 2015. Handbook of GC-MS: fundamentals and applications. John Wiley & Sons.

Inskipp, T., Gillett, H.J., 2005. Checklist of CITES species and annotated CITES appendices and reservations: a reference to the appendices to the Convention on International Trade in Endangered Species of Wild Fauna and Flora: United Nations Envir Programme.

Ismail, N., Rahiman, M.H.F., Taib, M.N., Ibrahim, M., Zareen, S., Tajuddin, S.N., 2016. Observation on SPME different headspace fiber coupled with GC-MS in extracting high quality agarwood chipwood. IEEE International Conference on Automatic Control and Intelligent Systems (I2CACIS) (pp. 214-218).

Jayachandran, K.S.I.P., Sekar, I., Parthiban, K.T., Amirtham, D., Suresh, K.K., 2014. Analysis of different grades of agarwood (*Aquilaria malaccensis* Lamk.) oil through GC-MS. *Indian Journal of Natural Products and Resources*, 5(1), 44-47.

Jensen, A., 2009. Valuation of non-timber forest products value chains. *Forest Policy and Economics*, 11(1), 34-41.

Jensen, A., Meilby, H., 2010. Returns from Harvesting a Commercial Non-timber Forest Product and Particular Characteristics of Harvesters and Their Strategies: *Aquilaria crassna* and Agarwood in Lao PDR1. *Economic Botany*, 64(1), 34-45.

Jung, D., 2011. The value of agarwood. Reflections upon its use and history in South Yemen.

Jung, D., 2013. The Cultural Biography of Agarwood–Perfumery in Eastern Asia and the Asian Neighbourhood1. *Journal of the Royal Asiatic Society*, 23(1), 103-125.

Kenmotsu, Y., Ogita, S., Katoh, Y., Yamamura, Y., Takao, Y., Tatsuo, Y., Kurosaki, F., 2011. Methyl jasmonate-induced enhancement of expression activity of Am-FaPS-1, a putative farnesyl diphosphate synthase gene from *Aquilaria microcarpa*. *Journal of Natural Medicines*, 65(1), 194-197.

Khan, A.L., Hamayun, M., Kim, Y.H., Kang, S.M., Lee, J.H., Lee, I.J., 2011. Gibberellins producing endophytic *Aspergillus fumigatus* sp. LH02 influenced endogenous phytohormonal levels, isoflavonoids production and plant growth in salinity stress. *Process Biochemistry*, 46(2), 440-447.

Lee, S.S., 2012. Checklist of fungi of Malaysia: Forest Research Institue Malaysia.

Leslie, J.F., Summerell, B.A., 2006. The fusarium laboratory manual. Ames, Iowa: Blackwell Publishing CrossRef Google Scholar, 388.

Li, X.J., Zhang, Q., Zhang, A.L., Gao, J.M., 2012. Metabolites from *Aspergillus fumigatus*, an endophytic fungus associated with *Melia azedarach*, and their antifungal, antifeedant, and toxic activities. *Journal of Agricultural and Food Chemistry*, 60(13), 3424-3431.

Liu, Y.Y., Wei, J.H., Gao, Z.H., Zhang, Z., Lyu, J.C., 2017. A review of quality assessment and grading for agarwood. *Chinese Herbal Medicines*, 9(1), 22-30.

Maffei, M.E., 2010. Sites of synthesis, biochemistry and functional role of plant volatiles. *South African Journal of Botany*, 76(4), 612-631.

Maggi, F., Papa, F., Cristalli, G., Sagratini, G., Vittori, S., 2010. Characterisation of the mushroom-like flavour of *Melittis melissophyllum* L. subsp. *melissophyllum* by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC–FID) and gas chromatography–mass spectrometry (GC–MS). *Food Chemistry*, 123(4), 983-992.

Mei, W.L., Yang, D.L., Wang, H., Yang, J.L., Zeng, Y.B., Guo, Z.K., Dai, H.F., 2013. Characterization and determination of 2-(2-phenylethyl) chromones in agarwood by GC-MS. *Molecules*, 18(10), 12324-12345.

Meng, Y., Pino, V., Anderson, J.L., 2011. Role of counteranions in polymeric ionic liquid-based solid-phase microextraction coatings for the selective extraction of polar compounds. *Analytica Chimica Acta*, 687(2), 141-149.

Mohamed, R., Jong, P.L., Nurul Irdayu, I., 2014. Succession patterns of fungi associated to wound-induced agarwood in wild *Aquilaria malaccensis* revealed from quantitative PCR assay. *World Journal of Microbiology and Biotechnology*, 30(9), 2427-2436.

Mohamed, R., Jong, P.L., Zali, M.S., 2010. Fungal diversity in wounded stems of *Aquilaria malaccensis*. *Fungal Diversity*, 43(1), 67-74.

Msaada, K., Hosni, K., Taarit, M.B., Chahed, T., Kchouk, M.E., Marzouk, B., 2007. Changes on essential oil composition of coriander (*Coriandrum sativum* L.) fruits during three stages of maturity. *Food Chemistry*, 102(4), 1131-1134.

Mulyaningsih, T., 2002. Formation of interxylary phloem and aromatic resin in *Gyrinops versteegii* (Thymelaeaceae). *IAWA Journal*, 23(4), 472-473.

Ng, L.T., Chang, Y.S., Kadir, A.A., 1997. A review on agar (gaharu) producing *Aquilaria* species. *Journal of Tropical Forest Products*, 2(2), 272-285.

Nguyen, T.T.T., 2014. Biodiversity of major bacterial groups in association with agarwood (*Aquilaria crassna*) in Khanh Hoa province, Vietnam. *Journal of Vietnamese Environment*, 6(2), 132-137.

Norhayati, M., Erneeza, M.H., Kamaruzaman, S., 2016. Morphological, pathogenic and molecular characterization of *Lasiodiplodia theobromae*: A causal pathogen of black rot disease on kenaf seeds in Malaysia. *International Journal of Agriculture & Biology*, 18(1), 80-85.

Novriyanti, E., Santosa, E., 2011. The role of phenolics in agarwood formation of *Aquilaria crassna* Pierre ex Lecomte AND Aquilaria microcarpa Baill Trees. *Indonesian Journal of Forestry Research*, 8(2), 101-113.

Novriyanti, E., Santosa, E., Syafii, W., Turjaman, M., Sitepu, I.R., 2010. Anti fungal activity of wood extract of *Aquilaria crassna* Pierre ex Lecomte against agarwood-inducing fungi, *Fusarium solani*. *Indonesian Journal of Forestry Research*, 7(2), 155-165.

Nurbaya, T.K., Baharuddin, A.R.D.S.M., 2014. Tentative identification of fungal isolates assosiated with *Aquillaria* spp. from remaining forest areas in Nunukan regency, North Kalimantan.
Parveen, Z., Siddique, S., Ali, Z., 2014. Chemical composition of essential oil from leaves of seeded and seedless *Citrus reticulata* Blanco var. *kinnow*. *Bangladesh Journal of Scientific and Industrial Research*, 49(3), 181-184.
Pavel, M., Radulescu, V., Ilies, D.C., 2009. GC-MS analysis of essential oil obtained from the species *Thymus comosus* Heuff. ex Griseb.(Lamiaceae). *Farmacia*, 57(4), 479-484.
PERsOOn, G.A., 2007. Agarwood: the life of a wounded tree.
Pinney, J.W., 2011. Host-pathogen systems biology. Handbook of statistical systems biology, 451-466.
Premalatha, K., Kalra, A.J.F.E., 2013. Molecular phylogenetic identification of endophytic fungi isolated from resinous and healthy wood of *Aquilaria malaccensis*, a red listed and highly exploited medicinal tree. *Fungal Ecology*, 6(3), 205-211.
Pripdeevech, P., Khummueng, W., Park, S.K., 2011. Identification of odor-active components of agarwood essential oils from Thailand by solid phase microextraction-GC/MS and GC-O. *Journal of Essential Oil Research*, 23(4), 46-53.
Putri, N., Karlinasari, L., Turjaman, M., Wahyudi, I., Nandika, D., 2017. Evaluation of incense-resinous wood formation in agarwood (*Aquilaria malaccensis* Lam.) using sonic tomography. *Agriculture and Natural Resources*, 51(2), 84-90.
Rahayu, G., 2010. Induction of agarwood formation by combination of acremonium and chemical treatments. http://repository.ipb.ac.id/handle/123456789/58961.
Ramli, A.N.M., Yusof, S., Bhuyar, P., Aminan, A.W., Tajuddin, S.N., Hamid, H.A., 2022. Production of Volatile Compounds by a Variety of Fungi in Artificially Inoculated and Naturally Infected *Aquilaria malaccensis*. *Current Microbiology*, 79(5), 1-14.
Saikia, P., Khan, M.L., 2012. Agar (*Aquilaria malaccensis* Lam.): a promising crop in the homegardens of Upper Assam, northeastern India. *Journal of Tropical Agriculture*, 50(1), 8-14.
Sangareswari, M., Parthiban, K.T., Kanna, S.U., Karthiba, L., Saravanakumar, D., 2016. Fungal microbes associated with agarwood formation. *American Journal of Plant Sciences*, 7(10), 1445.
Santoso, E., Irianto, R.S.B., Turjaman, M., Sitepu, I.R., 2011. Gaharu-producing tree induction technology. Proceeding of Gaharu Workshop, Development of Gaharu Production Technology, (pp. 31-45).
Satdive, R.K., Fulzele, D.P., Eapen, S., 2007. Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* A. Juss by elicitation and media optimization. *Journal of Biotechnology*, 128(2), 281-289.
Sen, S., Dehingia, M., Talukdar, N.C., Khan, M., 2017. Chemometric analysis reveals links in the formation of fragrant bio-molecules during agarwood (*Aquilaria malaccensis*) and fungal interactions. *Scientific Reports*, 7(1), 44406.
Shahnazi, S., Meon, S., Vadamalai, G., Ahmad, K., Nejat, N., 2012. Morphological and molecular characterization of *Fusarium* spp. associated with yellowing disease of black pepper (*Piper nigrum* L.) in Malaysia. *Journal of General Plant Pathology*, 78(3), 160-169.
Sulaiman, R., Thanarajoo, S.S., Kadir, J., Vadamalai, G., 2012. First report of *Lasiodiplodia theobromae* causing stem canker of *Jatropha curcas* in Malaysia. *Plant Disease*, 96(5), 767.
Sumadiwangsa, S., 1997. Agarwood as a high-value commodity in East Kalimantan [Kayu gaharu komoditi elit di Kalimantan Timur]. *Duta Rimba*, 20(205/206), 33-40.
Sumarna, Y., 2005. Strategi Budidaya dan Pengembangan Produksi Gaharu. In Prosiding Seminar Nasional Gaharu, Seameo-Biotrop, Bogor (pp. 1-2).
Tamuli, P., Baruah, P., Samanta, R., 2011. Enzyme activities of agarwood (*Aquilaria malaccensis* Lamk.) stem under pathogenesis. *Journal of Spices and Aromatic Crops*, 17(3), 240-243.
Taniguchi, S., Miyoshi, S., Tamaoki, D., Yamada, S., Tanaka, K., Uji, Y., Gomi, K., 2014. Isolation of jasmonate-induced sesquiterpene synthase of rice: product of which has an antifungal activity against *Magnaporthe oryzae*. *Journal of Plant Physiology*, 171(8), 625-632.
Tarigan, M., Wingfield, M.J., Van Wyk, M., Tjahjono, B., Roux, J., 2011. Pruning quality affects infection of *Acacia mangium* and *A. crassicarpa* by *Ceratocystis acaciivora* and *Lasiodiplodia theobromae*. *Southern Forests: a Journal of Forest Science*, 73(3-4), 187-191.
Tian, J.J., Gao, X.X., Zhang, W.M., Wang, L., Qu, L.H., 2013. Molecular identification of endophytic fungi from *Aquilaria sinensis* and artificial agarwood induced by pinholes-infusion technique. *African Journal of Biotechnology*, 12(21).
Tsan, P.L.J.P., Mohamed, R., 2014. Gas chromatography-mass spectrometry analysis of agarwood extracts from mature and juvenile *Aquilaria malaccensis*. *International Journal of Agriculture and Biology*, 16(3), 644-648.
Vichi, S., Castellote, A.I., Pizzale, L., Conte, L.S., Buxaderas, S., Lopez-Tamames, E., 2003. Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. *Journal of Chromatography A*, 983(1-2), 19-33.
Wang, S., Nomura, N., Nakajima, T., Uchiyama, H., 2012. Case study of the relationship between fungi and bacteria associated with high-molecular-weight polycyclic aromatic hydrocarbon degradation. *Journal of Bioscience and Bioengineering*, 113(5), 624-630.
Wiriadinata, H., Semiadi, G., Darnaedi, D., Waluyo, E.B., 2010. Konsep Budidaya gaharu (*Aquilaria* spp.) di Provinsi Bengkulu. *Jurnal Penelitian Hutan dan Konservasi Alam*, 7(4), 371-380.
Wu, B., Lee, J.G., Lim, C.J., Jia, S.D., Kwon, S.W., Hwang, G.S., Park, J.H., 2012. Sesquiterpenoids and 2-(2-Phenylethyl)-4H-chromen-4-one (=2-(2-Phenylethyl)-4H-1-benzopyran-4-one) Derivatives from *Aquilaria malaccensis* Agarwood. *Helvetica Chimica Acta*, 95(4), 636-642.
Zhang, C.L., Zheng, B.Q., Lao, J.P., Mao, L.J., Chen, S.Y., Kubicek, C.P., Lin, F.C., 2008. Clavatol and patulin formation as the antagonistic principle of *Aspergillus clavatonanicus*, an endophytic fungus of *Taxus mairei*. *Applied Microbiology and Biotechnology*, 78(5), 833-840.
Zhang, Z., Yang, Y., Meng, H., Sui, C.H., Wei, J.H., Chen, H.Q., 2010. Advances in studies on mechanism of agarwood formation in *Aquilaria sinensis* and its hypothesis of agarwood formation induced by defense response. *Chinese Traditional and Herbal Drugs*, 41(1), 156-159.
Zhang, Z., Zhang, X., Yang, Y., Wei, J.H., Meng, H., Gao, Z.H., Xu, Y.H., 2014. Hydrogen peroxide induces vessel occlusions and stimulates sesquiterpenes accumulation in stems of *Aquilaria sinensis*. *Plant Growth Regulation*, 72(1), 81-87.
Zviely, M., Li, M., 2013. Sesquiterpenoids: the holy fragrance ingredients. *Perfumer Flavorist*, 38, 52-55.

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RESEARCH ARTICLE

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Docking-based virtual screening, ADMET, and network pharmacology prediction of anthocyanidins against human alpha-amylase and alpha-glucosidase enzymes as potential antidiabetic agents

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ABSTRACT

Diabetes mellitus (DM) characterized by high blood sugar concentration is a major global public health problem and untreated DM results in blindness, kidney failure, heart attack, stroke, and lower extremity amputation. In this structure-based drug design (SBDD) study, the potential inhibitory effects of twelve anthocyanidins (aglycon unit of anthocyanins) components on human pancreatic α-amylase and intestinal α-glucosidase enzymes were investigated using the molecular docking method and a novel approach developed by our research group was used to rank the global binding potentials of ligands to a series of different enzymes simultaneously. In addition, drug-likeness, absorption-distribution-metabolism-excretion-toxicity (ADMET) predictions, and intracellular target-component interaction network analyses of twelve anthocyanidin components were performed using the search tool for interactions of chemicals (STITCH). Petunidin, peonidin, and aurantinidin were determined as 'hit' phytochemicals according to the docking binding energy and relative binding capacity index (RBCI) analyses, whereas, based on the RBCI index, petunidin was found to be the most effective ligand in terms of binding capacity to both enzymes that play an important role in DM. The more accessible and large-volume active site of α-amylase compared to α-glucosidase caused petunidin to bind with higher affinity against α-amylase. Promisingly, petunidin did not violate any of the criteria for drug-likeness consisting of a combination of the Lipinski's rule of 5, Ghose and Veber filters, showed no cytochrome (CYP) P450 or hERG I-II inhibitory activity in the ADMET analysis, however, it was found to have a low gastrointestinal absorption profile. In intracellular target-component network analysis using the STITCH online platform, it was determined that petunidin did not show negative functional interactions with any enzyme in the human protein network. Considering these results, it is recommended that petunidin be advanced to further *in vitro* and *in vivo* assays as a potential α-amylase and α-glucosidase inhibitory agent in the treatment of DM. However, the intestinal absorption profile of petunidin must be enhanced by molecular optimization without compromising its pharmacological activity.

List of Abbreviations

DM	: Diabetes mellitus
SBDD	: Structure-Based Drug Design
RBCI	: Relative Binding Capacity Index
ADMET	: Absorption-Distribution-Metabolism-Excretion-Toxicity
STITCH	: Search Tool for Interactions of Chemicals
CYP	: Cytochrome
IDF	: International Diabetes Federation
GDM	: Gestational Diabetes mellitus
T2DM	: Type 2 Diabetes mellitus
DFT	: Density Functional Theory
B3LYP	: Becke, 3-parameter, Lee–Yang–Parr
ATB	: Automated Topology Builder
PDB	: Protein data bank
NAMD	: Nanoscale Molecular Dynamics
Ro5	: Lipinski's rule of 5

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ClogP : Consensus octanol-water partition coefficient
Caco-2 : Caucasian colon adenocarcinoma cell line
VDss : Steady-state volume of distribution
logBB : Logarithmic ratio between the concentration of a compound in the brain and blood
logPS : The blood-brain permeability-surface area product
BBB : Blood Brain Barrier
CNS : Central Nervous System
OCT2 : Renal organic cation transporter 2
pkCSM : Predicting small-molecule pharmacokinetic properties using graph-based signatures
MRTD : Maximum recommended tolerated dose
hERG : Human ether-a-go-go gene
LD_{50} : Lethal dose 50
LOAEL : Lowest observed adverse effect level
$pIGC_{50}$: Negative logarithm of the concentration required to inhibit 50% growth in log µg/ml
LC_{50} : Lethal concentration 50

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder and is diagnosed with an elevated glucose concentration in the blood above the basal level. Among the clinical signs of DM, insulin secretion is observed to be generally defective, and the peripheral effects of insulin are weakened. In addition, defective metabolism of lipids, proteins, mineral salts, or electrolytes may accompany DM (Pérez Gutierrez et al., 2006). The International Diabetes Federation (IDF) reported that approximately 415 million adults aged 20 to 79 years had diabetes in 2015 (Zheng et al., 2018). This number, which is expected to increase by 200 million by 2040, is predicted as a global public health burden (Zheng et al., 2018). Although chronic hyperglycemia in DM patients causes damage to various organ systems, the most prominent of these are micro and macrovascular complications that increase cardiovascular risk. The incidence of DM is increasing globally due to the increase in lifestyles within the framework of unhealthy living conditions (obesity, wrong eating habits, physical inactivity, alcohol consumption, or smoking) (Chen et al., 2012; Forouhi and Wareham, 2014). The most prominent evidence that DM increases with age are that approximately 25% of the population over 65 years of age has diabetes (Carrillo-Larco et al., 2019).

Based on etiology and clinical findings, DM is classified into three categories: type 1 diabetes, type 2 diabetes, and gestational diabetes (GDM). Type 2 diabetes is found in the majority (90%) of all diabetes cases (Oliveira et al., 2020). In T2DM, the effect of insulin as a glucose utilization regulator is diminished, and this medical phenomenon is termed 'insulin resistance'. In the case of insulin resistance, insulin in the organism cannot function, a situation that contradicts the increased insulin production that initially occurs to maintain glucose homeostasis. Over time, insulin production decreases and T2DM develops. The prevalence of DM is most common in people older than 45 years. However, due to increasing obesity, physical inactivity, and energy-dense diets, DM has been increasingly diagnosed in children, adolescents, and young adults (Oliveira et al., 2020).

Phytochemicals offer a unique option for the discovery of new antidiabetic compounds with fewer side effects (Jayawardena et al., 2012). In this context, the combined use of plant-based compounds containing trace elements such as chromium or zinc arouses great interest in the scientific community, because activation of certain processes in glucose homeostasis and insulin sensitivity (activation of insulin receptor signaling pathway by chromium, the antioxidant activity of selenium and zinc) or enzymatic inhibition (inhibition of phosphatases by selenium) is thought to be promising (Wiernsperger and Rapin, 2010).

Therefore, various alkaloids, phenolic compounds, saponins, polysaccharides, terpenoids, glycosides, and xanthones that people take into the body by consuming different foods are important bioactive compounds (Coman et al., 2012).

Anthocyanins, a subcategory of dietary flavonoids, are water-soluble pigments that produce quite different colors (red, pink, purple, orange, and blue) in plants, fruits, vegetables, and leaves (Castañeda-Ovando et al., 2009; Wallace and Giusti, 2015). Anthocyanins are abundant in many vegetables, fruits, and tuber-type metamorphic vegetables (Belwal et al., 2017). Furthermore, anthocyanin content and composition vary widely in plant foods, especially fruits with high anthocyanin concentrations (Horbowicz et al., 2008). Anthocyanidins, aglycone forms of anthocyanins, are structured based on the 2-phenylbenzopyrilium or flavylium ions and carry methoxy or hydroxyl groups at different positions (Sivamaruthi et al., 2018). The human diet contains six major anthocyanidins (aglycone unit of anthocyanins), cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin, however, there are also anthocyanidins such as aurantinidin, capensinidin, europinidin, hirsutidin, pulchellidin, and rosinidin identified in nature. Color is a general property of anthocyanins/anthocyanidins and research indicates that it can be important in dietary guidance as certain anthocyanins/anthocyanidins and/or their metabolites have specific effects in reducing the risk of T2DM (Liu et al., 2022; Peterson et al., 2015; Zamora-Ros et al., 2011).

In this study, it was aimed to investigate the inhibitory potentials of certain anthocyanidin molecules (aurantinidin, capensinidin, cyanidin, delphinidin, europinidin, hirsutidin, malvidin, pelargonidin, peonidin, petunidin, pulchellidin, and rosinidin) (Figure 1) commonly found in plants on human pancreatic α-amylase and intestinal α-glucosidase (maltase glucoamylase) using the molecular docking method. Furthermore, drug-likeness properties, ADMET profiles, and *Homo sapiens* component-target interaction network of these compounds were also determined. It has been suggested that the hit anthocyanidin compounds that we determined in this study may be advanced to preclinical *in vitro* and *in vivo* studies to accelerate DM treatment.

2. Materials and methods

2.1. Structural optimization of ligands

Before molecular docking studies, the geometric optimization of 12 anthocyanidin molecules (ligands) was performed on the Automated Topology Builder (ATB) server (https://atb.uq.edu.au/) using the density functional theory (DFT/B3LYP/6-31G* basis set). After the geometry optimization of ligands, the energetically optimized 3D structures calculated by the ATB server were saved in pdb (protein data bank) file format before submission to molecular docking simulations (Malde et al., 2011).

2.2. Retrieval of α-amylase and α-glucosidase proteins and their structural optimization using nanoscale molecular dynamics (NAMD)

In the current study, the X-ray crystal structures of human α-amylase (PDB ID: 1B2Y) and α-glucosidase (maltase glucoamylase) (PDB ID: 3TOP) are downloaded from the Protein Data Bank (https://www.rcsb.org/) (RCSB PDB). Water molecules, co-

crystallized ligands, and non-interacting ions were removed from the protein structures before docking. Before energy minimization with NAMD (Nanoscale Molecular Dynamics), missing amino acid side chains and hydrogen atoms of protein molecules were added using the UCSF Chimera program. Then, in the Vega ZZ program, pancreatic α-amylase was ionized at pH 7.0, whereas the intestinal α-glucosidase was ionized at pH 5.0 (Sky-Peck and Thuvasethakul, 1977; Tomasik and Horton, 2012). In protein-energy minimization with NAMD, the atom types and electrical charges of the enzymes were fixed in the Vega ZZ program using the CHARMM22_PROT force field and Gasteiger-Marsili charges (Pedretti et al., 2004). Then, parameters required for energy minimization of proteins were loaded from a template file in the NAMD module of the same software. The value 10.000 was determined as the number of time steps, and the CHARMM22_PROT as the force field. Following the energy minimization, the lowest energy conformers (the last minimization step) of both proteins were recorded in protein data bank (pdb) format.

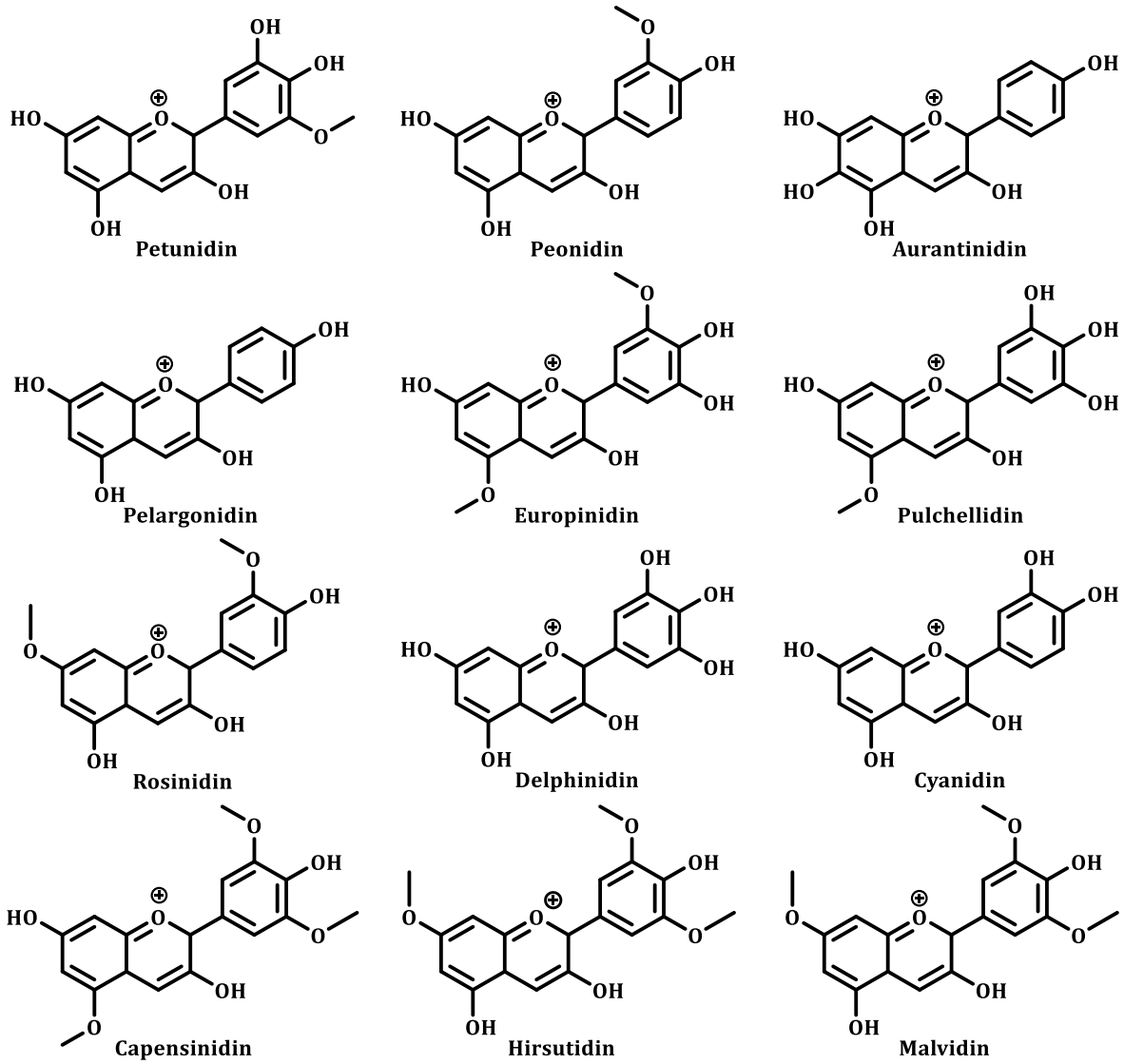

Figure 1. Chemical structures of anthocyanidins

2.3. Molecular docking

Molecular docking simulations between α-amylase and α-glucosidase (maltase glucoamylase) enzymes and anthocyanidins were performed using AutoDock Vina 1.2.0 program (Trott and Olson, 2010). In this rigid receptor-flexible ligand docking protocol, the binding affinity and binding conformations of anthocyanidins against the active sites (inhibitor binding site) of α-amylase and α-glucosidase enzymes were predicted with the AutoDock Vina 1.2.0 program. The AutoDockTools-1.5.6 interface has been used to prepare receptors and ligands before molecular docking experiments (Sanner, 1999). In molecular docking studies, the coordinates of the search spaces of the protein active sites were determined to allow ligands to interact easily with these regions. The determination of the active sites of α-amylase and α-glucosidase was performed using the cartesian coordinates of the bound experimental inhibitor (acarbose).

Using this approach, α-amylase active site coordinates were set as, x: 18.90, y: 5.79, z: 47.00 (size x: 28; size y: 28; size z: 28), and the α-glucosidase active site were set as, x: -55.97, y: 8.45, z: -64.73 (size x:30; size y:30, size z:30) accordingly.

After a total of 20 independent docking runs for each anthocyanidin, all possible ligand conformations were clustered by AutoDock Vina 1.2.0 according to geometric similarity and ranked based on the most favorable (negative) binding free energy (kcal/mol). Top-ranked poses of receptor-ligand complexes obtained with AutoDock Vina 1.2.0 were visualized using BIOVIA DS Visualizer v16 and examined for anthocyanidin-amino acid non-bonded interactions.

2.4. Determination of the relative binding capacity index (RBCI) of phytochemicals

In this study, 'Relative Binding Capacity Index (RBCI)' analysis, which is a new approach in the literature and enables a more efficient ranking of different ligands in molecular docking based on the receptors to which they show affinity, was performed (Istifli et al., 2022). RBCI provides a comparison of statistically relevant data that can have different scientific meanings. Because the binding affinities of ligands are different for each protein, phytochemicals can only be evaluated based on their potency in that parameter, if they are ranked in the light of their binding energies to a single protein. However, ranking based on only one of the receptors may not fully represent the activity potentials of the ligands. One of the most common methods used to rank interactions between receptor and ligand is to calculate the 'central bias'. In this method, ligands (components) are ranked based on the average value of their affinity for all receptors. If the values (binding free energy) in each data set are converted into standard scores, it is possible to compare these values with each other. Therefore, in this study, arithmetic mean and standard deviation were obtained for each protein by using binding free energy values of ligands. The raw standard scores were obtained by subtracting the binding free energy values of each ligand for each protein from this arithmetic mean and then dividing by the standard deviation value (see equation given below) (Sharma, 1996). The RBCI values of the ligands were obtained by calculating the averages of the standard scores (for each protein) as described above.

$$Standard\ score = (x-\mu)/\sigma$$

In the formula, 'x' stands for raw data, 'μ' stands for mean and 'σ' stands for standard deviation. Although RBCI is a relative value and does not fully represent the specific binding capacity of ligands, it makes possible to acceptably rank ligands according to their binding free energy values against all target receptors. Thus, RBCI could be viewed as an integrated approach to statistically rank order ligands based on their molecular interactions with different receptors since it considers the binding affinity for all receptors simultaneously.

2.5. Drug-likeness and absorption-distribution-metabolism-excretion-toxicity (ADMET) prediction

Determination of drug similarity (drug-likeness) and ADMET (absorption-distribution-metabolism-excretion-toxicity) profiles of promising hit compounds in structure-based drug design (SBDD) studies is important in terms of reducing their side effects on the target organism. In this work, SwissADME and pkCSM platforms were employed to predict drug-likeness and ADMET properties of anthocyanidins, for which docking simulations were performed against α-amylase and α-glucosidase enzymes (Daina et al., 2017; Daina et al., 2019; Pires et al., 2015).

2.6. Network pharmacology analysis

In the drug discovery process, the single drug/single target approach has some shortcomings in terms of safety and effectiveness (Chandran et al., 2015). Therefore, the application of a network pharmacology approach is necessary and inevitable in predicting the interactions of small molecules with the protein network of the relevant organism, revealing possible side effects of 'hit' or 'lead' compounds, or in the elucidation of novel therapeutic effects. In our study, component-target analysis of anthocyanidin molecules, which were determined as 'hit' according to the RBCI analysis, was performed using the STITCH (http://stitch.embl.de/) platform where '*H. sapiens*' was selected as the target organism.

3. Results and discussion

In this computational virtual screening study, the inhibitory potentials of twelve naturally occurring anthocyanidins (aglycone units of anthocyanins) (Table 1) against human pancreatic α-amylase and intestinal α-glucosidase (maltase-glucoamylase) enzymes were investigated using molecular docking method. The binding free energy (ΔG° = kcal/mol) calculated as a result of molecular docking was used as a criterion in the evaluation of the inhibition potentials of anthocyanidins on these enzymes.

Table 1. PubChem CID, molecular weight, and molecular formulas of anthocyanidins

No	Molecule	PubChem CID	Molecular weight (g/mol)	Molecular formula
1	Aurantinidin	441648	287.24	$C_{15}H_{11}O_6^+$
2	Capensinidin	441658	345.30	$C_{18}H_{17}O_7^+$
3	Cyanidin	128861	287.24	$C_{15}H_{11}O_6^+$
4	Delphinidin	68245	338.69	$C_{15}H_{11}ClO_7$
5	Europinidin	14496547	331.30	$C_{17}H_{15}O_7^+$
6	Hirsutidin	441694	345.30	$C_{18}H_{17}O_7^+$
7	Malvidin	159287	331.30	$C_{17}H_{15}O_7^+$
8	Pelargonidin	440832	271.24	$C_{15}H_{11}O_5^+$
9	Peonidin	441773	301.27	$C_{16}H_{13}O_6^+$
10	Petunidin	73386	352.72	$C_{16}H_{13}ClO_7$
11	Pulchellidin	14496545	317.27	$C_{16}H_{13}O_7^+$
12	Rosinidin	441777	315.30	$C_{17}H_{15}O_6^+$

Source: https://pubchem.ncbi.nlm.nih.gov/

Anthocyanidins structurally originate from the flavylium cation, an oxonium ion ($C_5H_5O^+$), and the hydrogen atoms in their structures are replaced by various functional groups (Figure 2). Generally, the colors they carry as a function of ambient pH can change from red to purple, blue, or bluish green (Iacobucci and Sweeny, 1983; Khoo et al., 2017). Furthermore, anthocyanidins are an important subclass of polymethine dyes and flavonoids. The flavylium cation is a chromium cation with a phenyl group attached to the 2nd carbon atom, and chromenillium (also known as benzo pyrylium) is a bicyclic version of pyrylium. The positive charge can move around the molecule (Khoo et al., 2017) (Figure 2).

3.1. RBCI values and molecular docking binding energies of anthocyanidins

Molecular docking is a structure-based drug design method (Structure-Based Drug Design = SBDD) that predicts the energetically lowest energy binding conformation of small molecule ligands on the target protein (receptor) (Dos Santos et al., 2018; Meng et al., 2011).

Molecular simulations, in this work, were performed between receptor molecules (pancreatic α-amylase and intestinal α-glucosidase) and 12 anthocyanidin components (aurantinidin, capensinidin, cyanidin, delphinidin, europinidin, hirsutidin, malvidin, pelargonidin, peonidin, petunidin, pulchellidin, and rosinidin), and the binding free energy (ΔG° = kcal/mol) of each anthocyanidin ligand against the target proteins was calculated. The obtained top-ranked conformations of anthocyanidins were saved and the chemical bonds stabilizing the resulting protein-ligand complexes were characterized. As a result of the RBCI analysis (Figure 3), that was performed using the binding free energy values of the ligands for both enzymes, it was determined that the 'hit' ligands among the 12 anthocyanidins were petunidin, peonidin, and aurantinidin. Therefore, the docking results of petunidin, peonidin, and aurantinidin were discussed in this section. The docking binding free energies and inhibition constants (K_i) of petunidin, peonidin, and aurantinidin are given in Table 2.

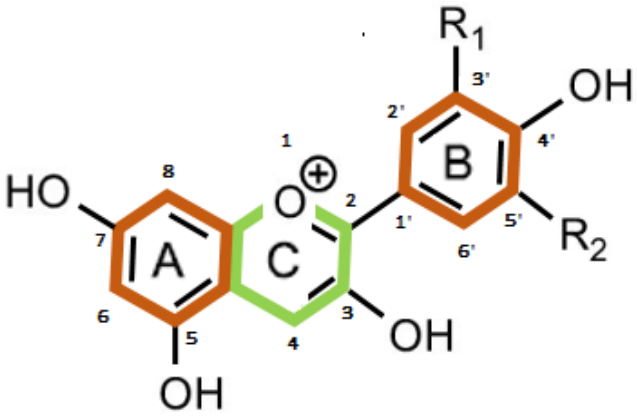

Figure 2. Structure of an anthocyanidin molecule.
The orange-colored aromatic rings are benzene (A and B), and the green-colored aromatic ring (C) is the pyrylium ring.
Anthocyanidins are named according to the specific groups at the R_1 and R_2 positions on the B ring

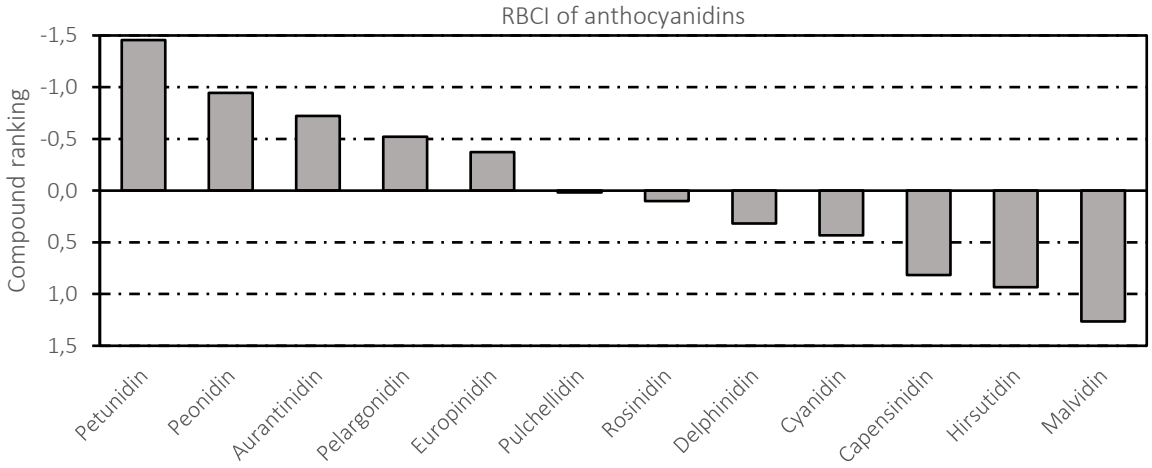

Figure 3. RBCI calculated using the binding free energy (kcal/mol) values of anthocyanidins against α-amylase and α-glucosidase enzymes.
The increase in the negative value of RBCI indicates that the compound in question binds more strongly with the active sites of both target receptors (α-amylase and α-glucosidase).

According to Table 2, while peonidin showed the highest affinity for the pancreatic α-amylase enzyme (ΔG°= -8.16 kcal/mol), petunidin (ΔG°= -7.37 kcal/mol) was determined to be the most effective ligand against intestinal α-glucosidase. K_i values of petunidin, peonidin, and aurantinidin molecules were determined to be between 0.90 – 1.60 µM for α-amylase, and 3.70 – 9.50 µM for α-glucosidase, respectively. Given that a drug candidate hit compound typically exhibits activity at concentrations close to 1 µM (Krasiński et al., 2005; Pellecchia et al., 2004), the binding affinity values of petunidin, peonidin, and aurantinidin molecules to the active site of the pancreatic α-amylase, in particular, are promising (Table 2). One of the most likely reasons for the stronger binding of these 3 phytochemicals to α-amylase compared to α-glucosidase is that the solvent-exposed surface of the α-amylase active site is conformationally larger in volume and more accessible. To examine this structural difference between the two enzymes, the inhibitor binding sites of human α-amylase (PDB ID:1b2y) and α-glucosidase (PDB ID:3top) can be visualized in the RCSB PDB database.

Table 2. Binding free energy (ΔG°=kcal/mol) and inhibition constant (K_i: μM) values of the interaction between hit anthocyanidins and human pancreatic α-amylase and intestinal α-glucosidase (maltase-glucoamylase) enzymes

No	Molecule	Enzyme	Binding free energy (kcal/mol)	Inhibition constant (K_i=μM)	Enzyme	Binding free energy (kcal/mol)	Inhibition constant (K_i=μM)
1	Petunidin	α-Amylase	-7.86	1.60	α-Glucosidase	-7.37	3.70
2	Peonidin	α-Amylase	-8.16	0.90	α-Glucosidase	-6.82	9.50
3	Aurantinidin	α-Amylase	-7.92	1.50	α-Glucosidase	-7.02	6.80

3.2. Molecular interactions between hit anthocyanidins and target proteins

The intermolecular interactions of 'hit' anthocyanidins (petunidin, peonidin, and aurantinidin) with α-amylase and α-glucosidase enzymes are given in Table 3. Analysis and visualization were performed with Discovery Studio Visualizer v16 (Accelrys) software, and non-covalent interactions between hit anthocyanidins and target enzymes, as well as interacting key amino acid residues were determined (Figures 4, 5, and 6).

Table 3. Amino acid molecular contacts and chemical interactions between human pancreatic α-amylase (1B2Y) and intestinal α-glucosidase (3TOP) enzymes and top-ranked anthocyanidins

No	Molecule	Enzyme	Classical H-bond	Van der Waals	Non-classical H-bond (C-H, Pi-Donor)	Hydrophobic interaction		Electrostatic
						π-π	Mixed π/Alkyl	
1	Petunidin	α-Amylase	Gln62[1], His100[1], Asp196[1]	Trp57, Leu164[1], Arg194, Ala197, Glu232, His298, Asp299, His304[1]	-	Trp58[1], Tyr61[1]	-	-
2	Peonidin	α-Amylase	Tyr61[1]	Trp57, Gln62[1], His100[1], Leu164[1], Arg194, Asp196[1], His298, Asp299, His304	Trp58[1]	Trp58[1]	-	-
3	Aurantinidin	α-Amylase	Arg194, Asp196	Trp57, Trp58[1], Gln62, Leu161, Ala197, Glu232, His298, Asp299	-	Tyr61[1], His100[1]	Leu164[1]	Asp196[1]
1	Petunidin	α-Glucosidase	Asp198[2], Gln199[2]	Gln199, Tyr292, Trp396[2], Lys501, Phe600[2], Thr627, Ile628	-	Trp410[2], Phe601[2]	-	-
2	Peonidin	α-Glucosidase	Thr627	Asp198[2], Gln199[2], Tyr292, Trp396[2], Lys501, Ile628	-	Trp410[2], Phe601[2]	-	-
3	Aurantinidin	α-Glucosidase	Asp198[2], Thr627	Gln199[2], Pro200, Tyr292, Trp396[2], Lys501, Phe600[2], Ile628, Gly629	-	Trp410[2], Phe601[2]	-	-

[1] Active site residues of pancreatic α-amylase (PDB ID: 1B2Y) interacting with co-crystallized inhibitor (acarbose), (Trp58, Tyr61, Gln62, His100, Leu164, Asp196, Lys199, His200, Glu239, His304, Gly305)

[2] Active site residues of intestinal α-glucosidase (PDB ID: 3TOP) interacting with the co-crystallized inhibitor (acarbose) (Asp198, Gln199, Lys205, Trp396, Trp410, Phe468, Arg551, Asp567, Phe600, Phe601, Arg623, His625)

Note: In the protein preparation step using the Chimera program, the residue numbers were changed for both structures (1B2Y and 3TOP) due to the processing method of the pdb files by the DockPrep module. Thus, for the reader to find the same residues in the 1B2Y file (chain A) which are indicated in the above table for α-amylase, the number of the relevant residue should be increased by 1 (+1), and in the same way, for the 3TOP file (chain B), it is necessary to increase it by 959 (+959).

Petunidin formed H-bonds with Gln62, His100, and Asp196, π-π interactions with Trp58 and Tyr61, and many Van der Waals interactions with Trp57, Leu164, Arg194, Ala197, Glu232, His298, Asp299 and His304 of the active site of the pancreatic α-amylase (Table 3, Figure 4-A). Based on the structural analysis of the top-ranked docking pose, it could be determined that the molecular complex formed by petunidin and the α-amylase is generally stabilized by H-bonds, hydrophobic and weak electrostatic interactions. In addition, the OH groups in the A, C, and B rings of petunidin played an important role in the formation of the H-bonds (Figure 4-A).

The types of molecular interactions and top-ranked pose of petunidin in the inhibitor binding site of the human intestinal α-glucosidase enzyme are given in Table 3 and Figure 4-B, respectively. In the active site of human intestinal α-glucosidase, petunidin formed H-bonds with Asp198 and Gln199, hydrophobic π-π interactions with Trp410 and Phe601, and many Van der Waals interactions with Gln199, Tyr292, Trp396, Lys501, Phe600, Thr627 and Ile628 (Table 3, Figure 4-B). It can be inferred that the molecular complex formed between petunidin and the active site of the α-glucosidase enzyme is stabilized by H-bonds and hydrophobic interactions. The OH group on the B ring of petunidin plays an important role in the formation of H-bonds (Figure 4-B).

The types of molecular interactions of peonidin in the inhibitor binding pocket of the human pancreatic α-amylase and its top-ranked pose are given in Table 3 and Figure 5-A, respectively. Peonidin formed H-bonds and π-π interaction with Tyr61 and Trp58, and many Van der Waals contacts with Trp57, Gln62, His100, Leu164, Arg194, Asp196, His298, Asp299, and His304 within the binding pocket of human α-amylase (Table 3, Figure 5-A). It was determined that the molecular complex formed by peonidin with the active site of the α-amylase is generally stabilized by a large number of hydrophobic interactions and a small number of H-bonds. Furthermore, the OH group on the C ring of peonidin was effective in the formation of the classical H-bond (Figure 5-A).

The types of molecular interactions of peonidin in the inhibitor binding site of the human intestinal α-glucosidase and its top-ranked pose are given in Table 3 and Figure 5-B, respectively. Peonidin, within the binding pocket of α-amylase, formed H-bond with Thr627, hydrophobic π-π interaction with Trp410 and Phe601, and many Van der Waals interactions with Asp198, Gln199, Tyr292, Trp396, Lys501 and Ile628 (Table 3, Figure 5-B). It has been

determined that the molecular complex formed between peonidin and the active site of human α-glucosidase is generally stabilized by a large number of hydrophobic stacking interactions and one H-bond. The C (pyrylium) and B (benzene) rings of peonidin played an important role in the formation of hydrophobic interactions (Figure 5-B).

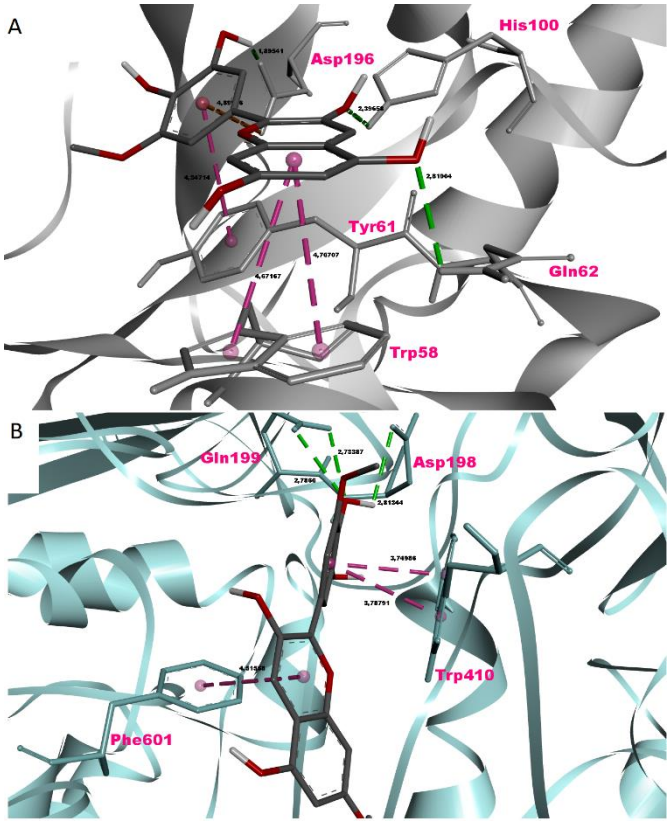

Figure 4. Top-ranked conformations of petunidin (A: α-amylase, B: α-glucosidase)

The types of molecular interactions of aurantinidin at the inhibitor binding site of the human pancreatic α-amylase and the top-ranked pose are given in Table 3 and Figure 6-A, respectively. Aurantinidin formed H-bond and electrostatic pi-anion interaction with Arg194 and Asp196, hydrophobic π-π and π-sigma contacts with Tyr61, His100, and Leu164, and many Van der Waals contacts with Trp57, Trp58, Gln62, Leu161, Ala197, Glu232, His298, and Asp299 with the α-amylase enzyme (Table 3, Figure 6-A). When the interaction of aurantinidin with the pancreatic α-amylase enzyme is examined, it is observed that the major interactions that stabilize the molecule in the active site of the enzyme are of hydrophobic type, but the H-bonds formed through the B ring also contributed to the stability of the complex (Figure 6-A).

The types of molecular interactions of aurantinidin in the inhibitor binding pocket of the human intestinal α-glucosidase and its top-ranked pose are given in Table 3 and Figure 6-B, respectively. Aurantinidin, in the active site of human intestinal α-glucosidase, formed two H-bonds with Asp198 and Thr627, hydrophobic π interactions with Trp410, Phe601, and Thr627, and numerous Van der Waals interactions with Gln199, Pro200, Tyr292, Trp396, Lys501, Phe600, Ile628, and Gly629 (Table 3, Figure 6-B). In general, it has been determined that the major interactions that stabilize aurantinidin in the active site of intestinal α-glucosidase are H-bonds and hydrophobic contacts (Figure 6-B).

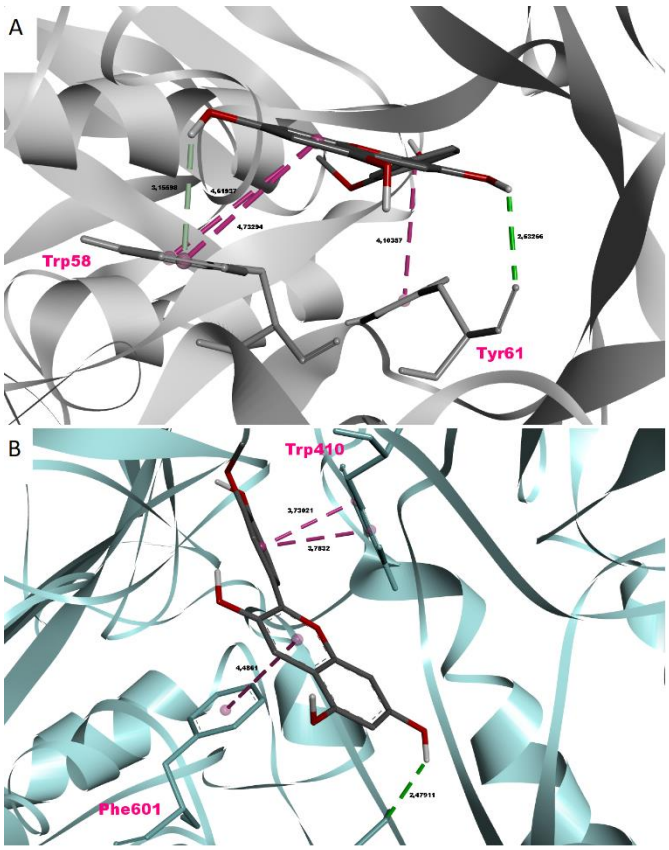

Figure 5. Top-ranked conformations of peonidin (A: α-amylase, B: α-glucosidase)

Based on a rigorous literature search, we found that there are no reports on the inhibitory potentials of petunidin, peonidin, and aurantinidin on human α-amylase or human α-glucosidase enzymes investigated by molecular docking. Therefore, our study is the first in the literature to determine the affinity between the above-mentioned ligands and human enzymes. On the other hand, since there are some docking or experimental inhibition studies of petunidin and peonidin against α-amylase or α-glucosidase enzymes obtained from non-human organisms, we compared and discussed our results with those from these studies.

It has been reported that the experimental IC_{50} value of petunidin against the α-glucosidase (maltase glucoamylase) enzyme from *Saccharomyces cerevisiae* was found to be 30.78 μM (K_i = 1.52 μM), with a mode of inhibition which was 'competitive' (Promyos et al., 2020). It was determined by the same researchers that petunidin showed a binding affinity of -7.98 kcal/mol against *S. cerevisiae* α-glucosidase enzyme in molecular docking simulations and formed non-bonded interactions with Asp327, Asp443, Asp542, and Tyr605 residues in the catalytic region of the enzyme. In general, the researchers found that the OH groups attached to the C-3', C-4', and C-5' carbons in the B ring (Figure 2) of an anthocyanidin molecule potentiate the inhibitory activity and that the OH group on the C-3' carbon of the B ring was more effective than the OH group attached to the C-5' carbon in terms of the inhibitory activity. Although the human-derived α-glucosidase enzyme was used as the target receptor in our study, petunidin interacted with Asp198 and Gln199

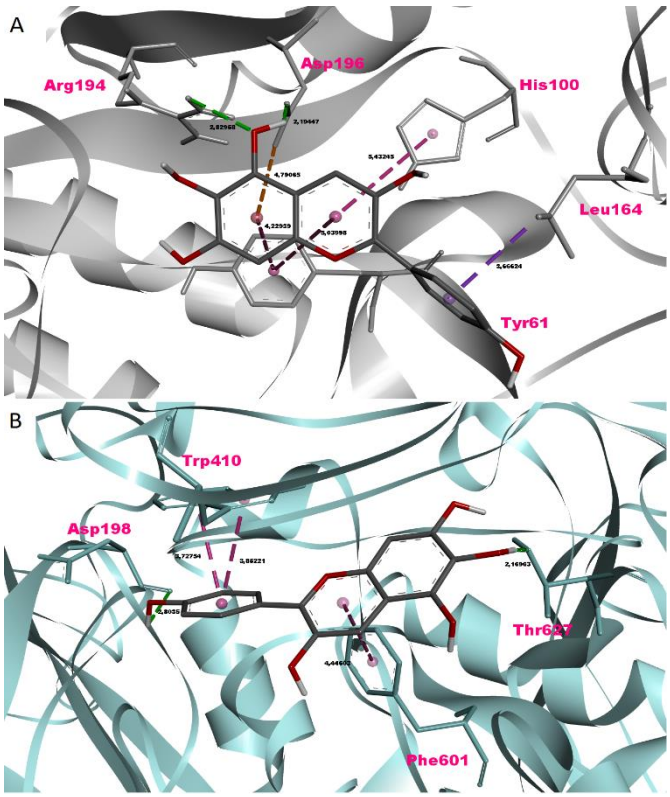

Figure 6. Top-ranked conformations of aurantinidin (A: α-amylase, B: α-glucosidase)

residues of the enzyme via the OH group attached to the C-4' carbon, but not the groups attached to the C-3' or C-5' carbons and formed 3 H-bonds (Figure 4-B). Therefore, the inhibitory reactions occurring in homologs of the same enzyme isolated from different organisms may be different, and the functional groups of the same ligand that interact with the active site residues may also differ. A low conservation value (5.34%) obtained as a result of the sequence alignment between *H. sapiens* and *S. cerevisiae* glucosidases is in line with this argument. These two homologous enzymes show great structural differences in terms of amino acid composition (Supplementary Figure S1). It has been reported that peonidin showed an IC_{50} of 36.11 µM (K_i = 1.23 µM) against the α-glucosidase isolated from *S. cerevisiae*, and its inhibition mode was found to be 'non-competitive' (Promyos et al., 2020). It was determined by the same researchers that peonidin displayed a binding strength of -6.40 kcal/mol against the α-glucosidase of *S. cerevisiae* in molecular docking simulations and formed chemical interactions with Asp203, Asp327, and Ser448 residues in the catalytic pocket of the enzyme (Promyos et al., 2020). In a different study, peonidin showed binding energy of -7.33 kcal/mol to the α-glucosidase enzyme isolated from the anaerobic gram-positive bacterium *Ruminococcus obeum*, and it was determined that this ligand formed chemical bonds with Asp73, Asp74, Ile76, Tyr85, Trp169, Asp197, Trp271, Asp307, Asp404, Trp417, Asp420, Lys422, and His478 residues of the enzyme (Zhang et al., 2019). In our study, peonidin formed contacts with the human α-glucosidase enzyme through residues Trp410, Phe601, and Thr627, and the C-7 carbon and B ring of the ligand (Figure 2) was effective in the formation of H-bond and hydrophobic contacts (Figure 5-B). As seen in our study and those of Zhang et al. (2019), peonidin could interact with polar and non-polar amino acid residues of α-glucosidase. Apolar (benzene and pyrylium rings) and polar (OH) functional groups of peonidin played an important role in these interactions (Figures 2 and 5-B).

Cyanidin, the other selected hit ligand in our docking study, was reported to show binding affinities of -8.18, -25.08 (*S. cerevisiae*), and -8.36 kcal/mol (*R. obeum*) against α-glucosidase enzyme isolated from *S. cerevisiae* and *R. obeum*, respectively (Chen et al., 2020; Promyos et al., 2020; Zhang et al., 2019). Delphinidin, another ligand for which docking calculations were performed in our study, has been reported to show binding capacities of -8.38 and -8.87 kcal/mol against α-glucosidase enzyme isolated from *S. cerevisiae* and *R. obeum*, respectively (Promyos et al., 2020; Zhang et al., 2019). On the other hand, malvidin and pelargonidin exhibited weak binding affinities of -3.97 and -4.69 kcal/mol against α-glucosidase enzyme isolated from *S. cerevisiae* (Promyos et al., 2020). As can be seen, cyanidin, delphinidin, pelargonidin, and malvidin, which were ranked lower according to the RBCI analysis against α-glucosidase (Figure 3), can show very high affinities against bacterial homologs of the same enzyme. However, the target enzymes (α-glucosidase and α-amylase) used in our docking study are of human origin and the amino acid residues in their catalytic sites have different characteristics in terms of both number and side chain structure (for example, the few residues in the active sites of *S. cerevisiae* α-glucosidase are predominantly composed of polar side chains, while the large number of active site residues of *H. sapiens* intestinal α-glucosidase consists of approximately equal amounts of polar and non-polar amino acids). Therefore, it was not possible to make a direct comparison with the studies of Chen et al. (2020), Promyos et al. (2020), and Zhang et al. (2019).

It has been determined that the chemical reactions of petunidin, peonidin, and aurantinidin in the catalytic sites of human α-amylase and α-glucosidase enzymes are generally stabilized by H-bonds, and a large number of hydrophobic π-π and Van der Waals interactions (Table 3). In addition, in contrast to the docking studies with *S. cerevisiae* in the literature (Promyos et al., 2020), all polar groups (-OH groups) and aromatic rings (A, C, or B) of the anthocyanidin molecule (Figure 2) play important roles to stabilize the ligand-receptor system (Figures 4, 5 and 6). In conclusion, petunidin, peonidin, and aurantinidin, which contain both polar and non-polar functional groups (Figures 1 and 2), show the ability to interact simultaneously with polar and non-polar side chains in the active sites of enzymes that play an important role in the digestive system, and therefore, they possess the ability to form stable complexes with human α-amylase and α-glucosidase enzymes.

3.3. Drug-likeness evaluation of top-ranked anthocyanidins

Drug-likeness can be defined as the molecular properties compatible with an adequate behavior in the body, namely good absorption (A), distribution (D), metabolism (M), excretion (E), and toxicity (T). Lipinski and co-workers have desired to identify, based on exclusion criteria, a formal and simplified list of molecular features that contribute most to drug similarity (Lipinski et al., 2012; Vistoli et al., 2008). In this context, 'Lipinski's rule of 5' (Ro5) attempts to elucidate broad chemical properties that can increase a molecule's chances of reaching the market (Shultz, 2018; Testa et al., 2007). On the other hand, although the popular Ro5 is quite effective in the discovery of new 'hit' or 'lead' compounds, only about 50% of chemical molecules discovered or synthesized in recent years fully comply with the Ro5 rule. In addition, some studies have shown that various organic compounds, such as macrolides and peptides, violate the chemical rules stipulated in the Ro5. Therefore, in addition to the Ro5 rule, variant filters such as the Ghose filter and the Veber rule have emerged in recent years (Congreve et al., 2003; Delaney, 2004; Ghose et al., 1999; Veber et al., 2002). Therefore, in this study, drug-likeness profiles of anthocyanidin molecules, which were docked with α-amylase and α-

glucosidase, were investigated by combining some filters such as Lipinski et al. (2012), Ghose et al. (1999), and Veber et al. (2002). The drug similarity features of these molecules (including top-ranked petunidin, peonidin, and aurantinidin) are given in Table 4.

Table 4. Drug-likeness profiles of anthocyanidins

No	Molecule	Number of H bond acceptors	Number of H bond donors	Number of rotatable bonds	TPSA[1]	Consensus Log *P*	Log *S* (ESOL[2])	Drug-likeness (Ro5)
1	Aurantinidin	6	5	1	114.29 Å^2	0.53	-3.34	Yes (0 violation)
2	Capensinidin	7	3	4	101.52 Å^2	1.37	-3.80	Yes (0 violation)
3	Cyanidin	6	5	1	114.29 Å^2	0.32	-2.60	Yes (0 violation)
4	Delphinidin	7	6	1	134.52 Å^2	-0.98	-3.16	Yes (1 violation; NH or OH > 5)
5	Europinidin	7	4	3	112.52 Å^2	1.05	-3.60	Yes (0 violation)
6	Hirsutidin	7	3	4	101.52 Å^2	1.16	-3.07	Yes (0 violation)
7	Malvidin	7	4	3	112.52 Å^2	0.92	-3.60	Yes (0 violation)
8	Pelargonidin	5	4	1	94.06 Å^2	0.93	-3.49	Yes (0 violation)
9	Peonidin	6	4	2	103.29 Å^2	0.97	-3.54	Yes (0 violation)
10	Petunidin	7	5	2	123.52 Å^2	-0.31	-4.10	Yes (0 violation)
11	Pulchellidin	7	5	2	123.52 Å^2	0.36	-2.66	Yes (0 violation)
12	Rosinidin	6	3	3	92.29 Å^2	1.20	-3.01	Yes (0 violation)

[1] TPSA: Topological polar surface area
[2] ESOL: Estimated aqueous solubility
Web source: http://www.swissadme.ch/index.php#

When Table 4 is evaluated, the docked 12 anthocyanidin molecules show acceptable properties for a potential drug-like molecule in terms of hydrogen bond acceptor and donor numbers, rotatable bond numbers, topological polar surface area (TPSA), and consensus LogP (ClogP) values. Though only the delphinidin molecule violates the Ro5 rule in terms of H bond donor number, since the number of these violations does not exceed 1, delphinidin could also be considered a potential drug-like entity. Petunidin, peonidin, and aurantinidin, determined as top-ranked 'hit' molecules according to RBCI analysis, do not violate the Ro5 rule based on any criteria. Among these three molecules, it is observed in Table 4 that the ligand with the lowest lipophilicity (oil solubility, ClogP) is petunidin. More polar and hydrophilic compounds tend to stay in the 'liquid phase'. Therefore, considering that most of the polar drugs enter the cell via transport from various membrane-embedded proteins (Kell et al., 2011), it can be assumed that petunidin is most probably the molecule with the highest potential to enter the cell via transport proteins on the cell membrane, as petunidin showed the most negative ClogP (-0.31) value (Table 4). Besides, a low LogP value (LogP < 4) and a low molecular weight (M.W. < 400) are more suitable for ADMET parameters (Gleeson, 2008). According to previous studies, it has been observed that the LogP values of molecules with the lowest toxicity risk and optimal ADME profile vary between 2-4 at pH 7.4 (Hann and Keserü, 2012; Lobo, 2020). Therefore, petunidin has, in our opinion, an excellent ClogP (-0.31) and molecular weight (352.72 g/mol, Table 1). Therefore, the ADMET evaluations in Table 5 were performed specifically for the promising molecule, petunidin.

3.4. ADMET profile of petunidin

The extensive data owned by pharmaceutical companies can conflict with the use of Ro5 and lead to conflicting situations. Therefore, early ADMET profiling of drug candidates is a crucial factor in understanding the potential therapeutic success of a new compound and can be effective in reducing unnecessary costs when integrated into the drug development process (Vistoli et al., 2008). According to Table 5, petunidin has a good water solubility value (-2.93 log mol/l) and slightly higher solubility in water than peonidin and aurantinidin. The Caco-2 monolayer cell line is widely used as an *in vitro* model of the human intestinal mucosa for predicting the absorption of orally administered drugs (Pires et al., 2015). High Caco-2 permeability is expressed by values greater than 0.90. However, since the estimated Caco-2 permeability of petunidin is -0.19 (< 0.90), it can be said that the probability of absorption from the human intestinal mucosa is low (Table 5). It is widely accepted that a molecule with an absorbance of less than 30% from the small intestine is poorly absorbed (Pires et al., 2015). For petunidin, this value is 68.96%, and therefore it was determined that petunidin's absorption from the small intestine is favorable (Table 5). However, considering the intestinal absorption value of other top-ranked ligand peonidin, which is 89.16%, this value (68.96%) determined for petunidin may not be reasonably high. A compound is considered to have a relatively low skin permeability if its logKp value is > -2.5. Petunidin was found to have a logKp of -2.73 (Table 5), and therefore the molecule's skin permeability is favorable. According to the calculation performed by the pkCSM online server, petunidin was determined to be a substrate of P-glycoprotein (P-gp) (Table 5). Inhibition of P-gp can increase the intracellular bioavailability of the drug molecule (Finch and Pillans, 2014). However, according to Table 5, it was also determined that the petunidin molecule was not an inhibitor of P-gp I or P-gp II. Taken together, petunidin may have the potential to be a P-gp activator. This may be an obstacle for petunidin to reach the sufficient intracellular concentration at which it can exert its pharmacological activity.

The steady-state volume of distribution (VDss) is the theoretical volume that a drug must be evenly distributed to the tissue to give the same concentration as detected in blood plasma. The higher the VD, the lower the plasma distribution of the drug, but the greater its distribution in the relevant tissue. If VDss is below 0.71 l/kg, it is considered low (log VDss < -0.15), and above 2.81 l/kg it is considered high (log VDss > 0.45) (Pires et al., 2015). In our study, the human VDss value of petunidin was determined to be 0.94. Therefore, it can be proposed that the estimated tissue distribution of petunidin is close to the baseline and possibly weak (Table 5). Associated with this data, the free fraction (fraction unbound) of petunidin in blood plasma was determined to be approximately 20% (Table 5). The ability of petunidin to cross the blood-brain barrier (BBB) was determined as -1.07 logBB (ratio of a drug's concentration in the brain to its plasma concentration), and a value less than -1 indicates that petunidin may not effectively cross the BBB (Table 5). The logPS value of petunidin was determined to be -3.88 (Table 5), indicating that the molecule could also not penetrate the central nervous system (CNS). However, the inability of petunidin to cross the BBB or CNS is not a major drawback, given that a potential anti-diabetic drug or hit molecule must act primarily on pancreatic or circulating glucose receptors.

When the estimated liver metabolism parameters of petunidin are examined, it is strikingly evident that petunidin is not a substrate or inhibitor of some cytochrome (CYP) P450 detoxification enzymes (Table 5). Not being used as a substrate by CYP enzymes indicates that petunidin is not metabolized in the liver, which is an advantageous profile for this ligand. In conclusion, petunidin, most likely, is not degraded in the liver and does not show an inhibitory effect on CYP enzymes that play a direct role in drug metabolism.

Table 5. Predicted ADMET profiles of anthocyanidins

	Model name	Aurantinidin	Capensinidin	Cyanidin	Delphinidin	Europinidin	Hirsutidin	Malvidin	Pelargonidin	Peonidin	Petunidin	Pulchellidin	Rosinidin	Unit
ABSORPTION	Water solubility	-2.96	-3.47	-2.93	-2.91	-3.22	-3.58	-3.07	-3.08	-3.05	-2.93	-2.92	-3.55	log mol/l
	Caco-2 permeability	-0.88	-0.08	-0.35	-0.32	-0.16	0.04	-0.38	-0.08	-0.13	-0.19	-1.02	0.98	log cm/S
	Intestinal absorbtion (human)	72.61	94.76	87.30	61.91	90.34	84.29	88.78	87.29	89.16	68.96	68.15	90.47	% absorbed
	Skin permeability	-2.73	-2.73	-2.73	-2.73	-2.73	-2.73	-2.73	-2.73	-2.73	-2.73	-2.73	-2.73	logK/p
	P-glycoprotein substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical
	P-glycoprotein I inhibitor	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	P-glycoprotein II inhibitor	No	Yes	No	No	No	Yes	No	No	No	No	No	Yes	Categorical
DISTRIBUTION	[1]VDss (human)	0.84	0.54	0.95	0.96	0.66	-0.21	0.76	0.64	0.56	0.94	0.86	-0.04	log l/kg
	Fraction unbound	0.25	0.07	0.24	0.33	0.09	0.05	0.13	0.20	0.10	0.20	0.20	0.03	Fu (fraction unbound)
	BBB[2] permeability	-1.61	-1.27	-1.23	-1.28	-1.34	-1.29	-1.35	-1.06	-1.25	-1.07	-1.67	-1.19	logBB
	CNS permeability	-2.26	-3.26	-2.21	-3.92	-3.36	-3.01	-3.37	-2.02	-2.29	-3.88	-3.20	-2.21	logPS
METABOLISM	CYP2D6[3] substrate	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	CYP3A4 substrate	No	Yes	No	No	No	Yes	No	No	Yes	No	No	Yes	Categorical
	CYP1A2 inhibitor	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Categorical
	CYP2C19 inhibitor	No	Yes	No	No	Yes	Yes	No	Yes	No	No	No	Yes	Categorical
	CYP2C9 inhibitor	No	No	No	No	Yes	Yes	No	No	Yes	No	No	Yes	Categorical
	CYP2D6 inhibitor	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	CYP3A4 inhibitor	No	Yes	No	No	No	No	No	No	No	No	No	No	Categorical
EXCRETION	Total clearance	0.47	0.76	0.53	0.57	0.72	0.746	0.68	0.576	0.63	0.64	0.61	0.70	log ml/min/kg
	Renal OCT2[4] substrate	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
TOXICITY	AMES toxicity	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	Max. tolerated dose (human)	0.55	0.23	0.49	0.50	0.40	0.65	0.55	0.50	0.56	0.52	0.51	0.54	log mg/kg/day
	hERG I[5] inhibitor	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	hERG II inhibitor	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	Rat acute oral toxicity (LD_{50})	2.49	11.72	2.46	2.54	2.34	11.72	2.34	2.43	2.40	2.45	2.54	13.91	mol/kg
	Rat chronic oral toxicity (LOAEL)	3.22	1.60	2.54	44.80	1.96	24.83	2.41	16.83	2.43	2.61	2.75	1.80	log mg/kg.bw/day
	Hepatotoxicity	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	Skin sensitization	No	No	No	No	No	No	No	No	No	No	No	No	Categorical
	T. pyriformis toxicity	0.28	0.35	0.29	0.28	0.35	0.34	0.32	0.31	0.31	0.29	0.29	0.35	log µg/l
	Minnow toxicity	1.94	1.02	2.54	31.10	2.51	1.62	1.22	1.81	1.40	3.00	2.03	1.18	log mM

[1] VDss: Volume of distribution
[2] BBB: Blood Brain Barrier
[3] CYP: Cytochrome P450
[4] OCT2: Organic Cation Transporter 2
[5] hERG I-II: Human Ether-à-go-go gene [KCNH2: potassium ion channel alpha-subunit (Kv11.1) coding gene]
Web source: http://biosig.unimelb.edu.au/pkcsm/

According to Table 5, the total clearance of petunidin is 0.64 ml/min/kg. Furthermore, petunidin is not a substrate of the renal organic cation transporter 2 (OCT2) (Table 5). OCT2 is a renal uptake protein that plays an important role in the regulation and renal clearance of drugs and endogenous compounds. OCT2 substrates may interact adversely with combined OCT2 inhibitors (Pires et al., 2015). The fact that petunidin is not an OCT2 substrate indicates that it does not adversely affect drug excretion processes in the kidney.

According to the pkCSM online server, the estimated AMES test result of petunidin is negative and therefore not mutagenic (Table 5). The maximum recommended tolerated dose (MRTD) provides an estimate of the threshold for toxic doses of chemicals in the human body. According to Table 5, the MRTD of petunidin is 0.52 mg/kg/day, and since this value is greater than 0.477, it was concluded that the MRTD of the petunidin molecule is favorably high. Hopefully, petunidin shows no inhibitory effect on potassium channels encoded by hERG I and hERG II (Table 5). Lethal dose values (LD_{50}) are standard values for measuring the relative acute toxicities of different molecules. The rat acute oral toxicity (LD_{50}) value for petunidin was calculated as 2.45 mol/kg, and the rat chronic oral toxicity (LOAEL) value was determined as 2.61 log mg/kg.bw/day. These values (LD_{50} and LOAEL) become meaningful when compared with the bioactive concentration of the molecule in the organism and the treatment time, however, in general, highly toxic (positive) chemicals have less than 50 mg/kg (LD_{50} < 50 mg/kg), and non-toxic (negative) chemicals, on the other hand, have

higher than 2000 mg/kg (LD_{50} > 2000 mg/kg) acute oral toxicity values (Gadaleta et al., 2019). In the computational conversion performed according to Table 5, the acute oral toxicity value of petunidin was found to be 778263.31 mg/kg. This value indicates that petunidin is a reliable phytochemical without oral toxicity. Petunidin, promisingly, does not show hepatotoxicity nor does it cause skin sensitization (Table 5). Finally, although petunidin is a potential toxic agent in *Tetrahymena pyriformis* ($pIGC_{50}$ = 0.29 µg/l), it shows no toxic effect in the minnow (LC_{50} = 3.00 log mM).

3.5. Network pharmacology analysis of anthocyanidins

The increase in the rate of drugs failing late in the clinical development process over the last decade stems from the assumption that the goal of drug discovery is to design selective ligands specifically designed for a single disease target. The underlying assumption of this current drug design approach is that safer, more effective drugs can be obtained through the development of highly selective ligands with eliminated undesirable toxic side effects. However, after nearly two decades of efforts to develop highly selective ligands, the high attrition rate of drugs in the design phase has begun to undermine this hypothesis (Hopkins, 2008; Szklarczyk et al., 2016). Today, the role of the ligand-protein interaction network is even more prominent in the field of drug development, as diseases are often the phenotypic result of multiple changes in the same pathway or multi-protein complex (Barabási et al., 2011; Oti et al., 2006). Taking into account the network in which the individual protein targeted by the ligand is located and the topology of this network can provide a better understanding of the cellular effect of a drug (Hopkins, 2008; Szklarczyk et al., 2016; Yang et al., 2008).

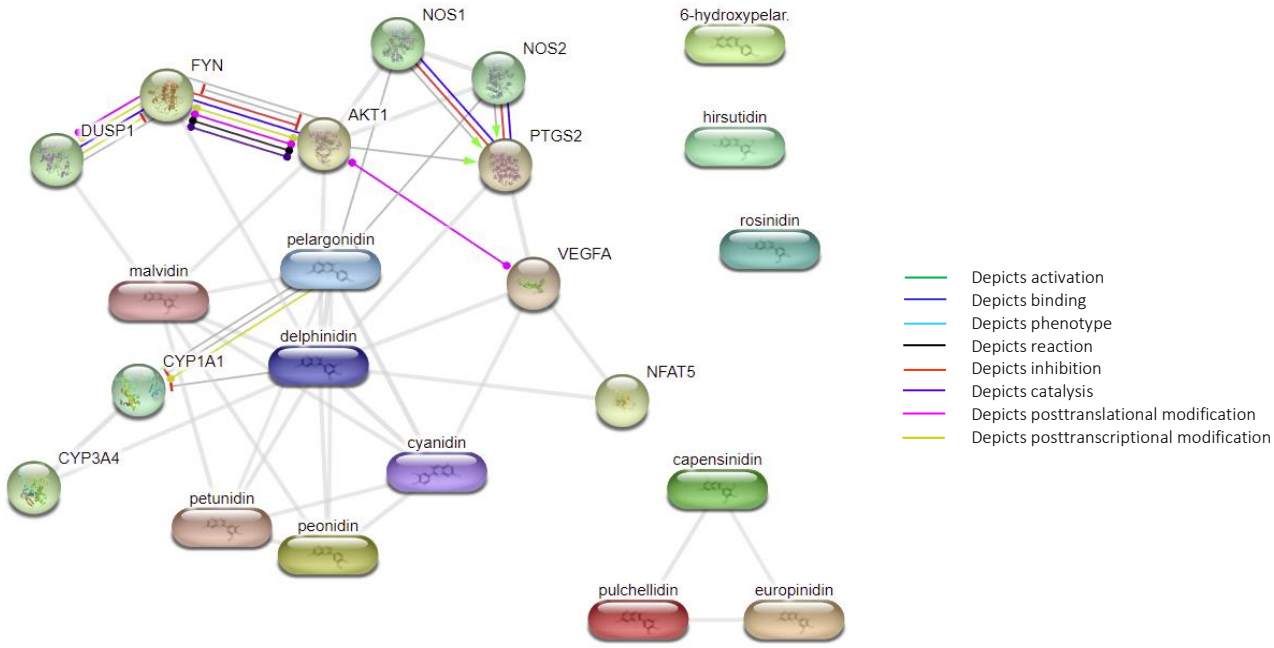

Figure 7. The 'molecular effect' view of the STITCH analysis results showing possible intracellular protein targets and interaction types of twelve anthocyanidin molecules

The minimum interaction score was set as 0.70 (high confidence).
Source: http://stitch.embl.de/cgi/network.pl?taskId=5zMVyBQC4MaY

In this context, the Search Tool for Interactions of Chemicals (STITCH) database was used to determine the interactions of 12 anthocyanidin molecules with their intracellular protein(s) targets and the types of these interactions. The 'minimum required interaction score was selected as 0.70 (high confidence) in the STITCH server, revealing the possible protein molecular interaction network of anthocyanidins in *H. sapiens* (Figure 7). According to Figure 7, the predicted *H. sapiens* functional protein partners of petunidin, peonidin, cyanidin, delphinidin, malvidin, and pelargonidin appear to be VEGFA, PTGS2, AKT1, NFAT5, FYN, CYP3A4, DUSP1, NOS1, NOS2, and CYP1A1. Furthermore, the anthocyanidins petunidin, peonidin, cyanidin, delphinidin, malvidin, and pelargonidin are highly associated, and delphinidin is at the center of this ligand network (Figure 7). While pelargonidin shows multiple effects such as activation, inhibition, and posttranscriptional regulation (combined score = 0.70) on CYP1A1, delphinidin shows an inhibitory effect (combined score = 0.73) on CYP1A1 protein (Figure 7). Therefore, it can be assumed that there could be a multifunctional interaction between pelargonidin, delphinidin, and CYP1A1. The petunidin molecule, which was determined as the top-ranked ligand in our study, promisingly does not exert any significant functional interaction with any protein in this molecular interaction network (Figure 7). The molecular interaction network properties for petunidin are also consistent with the ADMET results in Table 5; petunidin does not react in a positive or negative functional manner with any cytochrome P450 enzymes. In previous studies, it has been reported that anthocyanins showed a favorable effect on obesity and diabetes by increasing adipocytokine gene expression and secretion. Cyanidin, but not petunidin, altered the expression levels of this gene in human adipocytes, up-regulated adiponectin and leptin, and induced genes related to lipid metabolism (uncoupling protein 2, acyl-CoA oxidase 1, and perilipin) (Tsuda et al., 2006). Also, cyanidin potentiated the expression of PPAR and adipocyte-specific genes such as adipocyte fatty acid binding protein (aP2) and lipoprotein lipase (LPL) (Tsuda et al., 2004). Although these results do not include the hit ligands identified in our study, a similar mechanism could be suggested for petunidin or other anthocyanidins, since cyanidin is structurally similar to petunidin. In conclusion, these data imply that anthocyanidins may regulate adipocytokine gene expression through multiple mechanisms and provide a preventive effect on obesity and diabetes (Tsuda et al., 2005). However, it should be

noted that different anthocyanidins can affect the expression of different genes.

4. Conclusions

In this study, petunidin, an anthocyanidin phytochemical, showed a very favorable energetic binding potential in molecular docking simulations against human pancreatic α-amylase and intestinal α-glucosidase enzymes (-7.86 kcal/mol for α-amylase; -7.37 kcal/mol for α-glucosidase). The fact that the α-amylase active site is more solvent-accessible and has a large volume was evaluated as a factor that strengthens the interaction of petunidin with this enzyme. It has been determined that the hydrophobic interactions between the aromatic groups (A, C, and B rings) of petunidin and proteins' active site residues are at least as effective as the H-bonds in the interaction of petunidin with human α-amylase and α-glucosidase. According to drug-likeness analysis using the combined Ro5, Ghose, and Veber filters, petunidin showed promising 'hit' molecule properties and strikingly did not violate any physicochemical criteria. ADMET analysis indicated that petunidin was unlikely to be absorbed from the intestinal mucosa and its estimated tissue distribution was poor. On the other hand, petunidin does not interact negatively with liver metabolic enzymes, cytochrome (CYP) P450s, and does not act as a substrate for renal organic cation transporter 2 (OCT2). Very promisingly, petunidin showed no mutagenic potential, and no inhibition on hERG I and hERG II potassium channels, with a reliable LD_{50} profile. Furthermore, network pharmacology analysis showed that petunidin had no negative functional interactions with human intracellular target proteins. Based on molecular docking, drug-likeness, ADMET, and network pharmacology, petunidin can be suggested as a potential α-amylase and α-glucosidase inhibitor in postprandial hyperglycemia observed in T2DM patients. On the other hand, the low gastrointestinal absorption rate should be increased by molecular optimization without compromising petunidin's pharmacological efficacy.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Cihan Demir: Conceptualization, Data curation, Investigation, Methodology, Writing
Erman Salih Istifli: Data curation, Formal analysis, Methodology, Software, Visualization, Review & Editing

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Supplementary File

The supplementary file accompanying this article is available at https://ijpbp.com/index.php/ijpbp/libraryFiles/downloadPublic/10.

References

Barabási, A.L., Gulbahce, N., Loscalzo, J., 2011. Network medicine: a network-based approach to human disease. *Nature Reviews Genetics*, 12(1), 56-68.
Belwal, T., Nabavi, S.F., Nabavi, S.M., Habtemariam, S., 2017. Dietary anthocyanins and insulin resistance: When food becomes a medicine. *Nutrients*, 9(10), 1111.
Carrillo-Larco, R.M., Barengo, N.C., Albitres-Flores, L., Bernabe-Ortiz, A., 2019. The risk of mortality among people with type 2 diabetes in Latin America: A systematic review and meta-analysis of population-based cohort studies. *Diabetes/Metabolism Research and Reviews*, 35(4), e3139.
Castañeda-Ovando, A., de Lourdes Pacheco-Hernández, M., Páez-Hernández, M.E., Rodríguez, J.A., Galán-Vidal, C.A., 2009. Chemical studies of anthocyanins: A review. *Food Chemistry*, 113(4), 859-871.
Chandran, U., Mehendale, N., Tillu, G., Patwardhan, B., 2015. Network pharmacology of ayurveda formulation Triphala with special reference to anti-cancer property. *Combinatorial Chemistry & High Throughput Screening*, 18(9), 846-854.
Chen, J.G., Wu, S.F., Zhang, Q.F., Yin, Z.P., Zhang, L., 2020. α-Glucosidase inhibitory effect of anthocyanins from *Cinnamomum camphora* fruit: Inhibition kinetics and mechanistic insights through *in vitro* and *in silico* studies. *International Journal of Biological Macromolecules*, 143, 696-703.
Chen, L., Magliano, D.J., Zimmet, P.Z., 2012. The worldwide epidemiology of type 2 diabetes mellitus—present and future perspectives. *Nature Reviews Endocrinology*, 8(4), 228-236.
Coman, C., Rugina, O.D., Socaciu, C., 2012. Plants and natural compounds with antidiabetic action. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 40(1), 314-325.
Congreve, M., Carr, R., Murray, C., Jhoti, H., 2003. A'rule of three'for fragment-based lead discovery?. *Drug Discovery Today*, 8(19), 876-877.
Daina, A., Michielin, O., Zoete, V., 2017. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, 7(1), 1-13.
Daina, A., Michielin, O., Zoete, V., 2019. SwissTargetPrediction: updated data and new features for efficient prediction of protein targets of small molecules. *Nucleic Acids Research*, 47(W1), W357-W364.
Delaney, J.S., 2004. ESOL: estimating aqueous solubility directly from molecular structure. *Journal of Chemical Information and Computer Sciences*, 44(3), 1000-1005.
Finch, A., Pillans, P., 2014. P-glycoprotein and its role in drug-drug interactions. *Australian Prescriber*, 37(4), 137-139.
Forouhi, N.G., Wareham, N.J., 2014. Epidemiology of diabetes. *Medicine (Abingdon)*, 42 (12): 698–702.
Gadaleta, D., Vuković, K., Toma, C., Lavado, G.J., Karmaus, A.L., Mansouri, K., Roncaglioni, A., 2019. SAR and QSAR modeling of a large collection of LD_{50} rat acute oral toxicity data. *Journal of Cheminformatics*, 11(1), 1-16.
Ghose, A.K., Viswanadhan, V.N., Wendoloski, J.J., 1999. A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. *Journal of Combinatorial Chemistry*, 1(1), 55-68.
Gleeson, M.P., 2008. Generation of a set of simple, interpretable ADMET rules of thumb. *Journal of Medicinal Chemistry*, 51(4), 817-834.
Hann, M.M., Keserü, G.M., 2012. Finding the sweet spot: the role of nature and nurture in medicinal chemistry. *Nature Reviews Drug Discovery*, 11(5), 355-365.
Hopkins, A.L., 2008. Network pharmacology: the next paradigm in drug discovery. *Nature Chemical Biology*, 4(11), 682-690.
Horbowicz, M., Kosson, R., Grzesiuk, A., Debski, H., 2008. Anthocyanins of fruits and vegetables-their occurrence, analysis and role in human nutrition. *Vegetable Crops Research Bulletin*, 68, 5-22.
Iacobucci, G.A., Sweeny, J.G., 1983. The chemistry of anthocyanins, anthocyanidins and related flavylium salts. *Tetrahedron*, 39(19), 3005-3038.
Istifli, E.S., Netz, P.A., Sihoglu Tepe, A., Husunet, M.T., Sarikurkcu, C., Tepe, B., 2022. *In silico* analysis of the interactions of certain flavonoids with the receptor-binding

domain of 2019 novel coronavirus and cellular proteases and their pharmacokinetic properties. *Journal of Biomolecular Structure and Dynamics*, 40(6), 2460-2474.
Jayawardena, R., Ranasinghe, P., Galappatthy, P., Malkanthi, R.L.D.K., Constantine, G.R., Katulanda, P., 2012. Effects of zinc supplementation on diabetes mellitus: a systematic review and meta-analysis. *Diabetology & Metabolic Syndrome*, 4(1), 1-12.
Kell, D.B., Dobson, P.D., Oliver, S.G., 2011. Pharmaceutical drug transport: the issues and the implications that it is essentially carrier-mediated only. *Drug Discovery Today*, 16(15-16), 704-714.
Khoo, H.E., Azlan, A., Tang, S.T., Lim, S.M., 2017. Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food & Nutrition Research*, 61(1), 1361779.
Krasiński, A., Radić, Z., Manetsch, R., Raushel, J., Taylor, P., Sharpless, K.B., Kolb, H.C., 2005. *In situ* selection of lead compounds by click chemistry: target-guided optimization of acetylcholinesterase inhibitors. *Journal of the American Chemical Society*, 127(18), 6686-6692.
Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P. J. (2012). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews*, 64, 4-17.
Liu, Y., Wang, Q., Wu, K., Sun, Z., Tang, Z., Li, X., Zhang, B., 2022. Anthocyanins' effects on diabetes mellitus and islet transplantation. *Critical Reviews in Food Science and Nutrition*, 1-24.
Lobo, S., 2020. Is there enough focus on lipophilicity in drug discovery?. *Expert Opinion on Drug Discovery*, 15(3), 261-263.
Malde, A.K., Zuo, L., Breeze, M., Stroet, M., Poger, D., Nair, P.C., Mark, A.E., 2011. An automated force field topology builder (ATB) and repository: version 1.0. *Journal of Chemical Theory and Computation*, 7(12), 4026-4037.
Meng, X.Y., Zhang, H.X., Mezei, M., Cui, M., 2011. Molecular docking: a powerful approach for structure-based drug discovery. *Current Computer-Aided Drug Design*, 7(2), 146-157.
Oliveira, H., Fernandes, A., Brás, N., Mateus, N., de Freitas, V., Fernandes, I., 2020. Anthocyanins as antidiabetic agents—*in vitro* and *in silico* approaches of preventive and therapeutic effects. *Molecules*, 25(17), 3813.
Oti, M., Snel, B., Huynen, M.A., Brunner, H.G., 2006. Predicting disease genes using protein–protein interactions. *Journal of Medical Genetics*, 43(8), 691-698.
Pedretti, A., Villa, L., Vistoli, G., 2004. VEGA–an open platform to develop chemo-bio-informatics applications, using plug-in architecture and script programming. *Journal of Computer-Aided Molecular Design*, 18(3), 167-173.
Pellecchia, M., Becattini, B., Crowell, K.J., Fattorusso, R., Forino, M., Fragai, M., Tautz, L., 2004. NMR-based techniques in the hit identification and optimisation processes. *Expert Opinion on Therapeutic Targets*, 8(6), 597-611.
Pérez Gutierrez, R., Hernández Luna, H., Hernández Garrido, S., 2006. Antioxidant activity of *Tagetes erecta* essential oil. *Journal of the Chilean Chemical Society*, 51(2), 883-886.
Peterson, J.J., Dwyer, J.T., Jacques, P.F., McCullough, M.L., 2015. Improving the estimation of flavonoid intake for study of health outcomes. *Nutrition Reviews*, 73(8), 553-576.
Pires, D.E., Blundell, T.L., Ascher, D.B., 2015. pkCSM: predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures. *Journal of Medicinal Chemistry*, 58(9), 4066-4072.
Promyos, N., Temviriyanukul, P., Suttisansanee, U., 2020. Investigation of anthocyanidins and anthocyanins for targeting α-glucosidase in diabetes mellitus. *Preventive Nutrition and Food Science*, 25(3), 263-271.
Sanner, M.F., 1999. Python: a programming language for software integration and development. *Journal of Molecular Graphics and Modelling*, 17(1), 57-61.
Santos, R.N.D., Ferreira, L.G., Andricopulo, A.D., 2018. Practices in molecular docking and structure-based virtual screening. In Computational drug discovery and design (pp. 31-50). Humana Press, New York, NY.
Sharma, S. (1996). Applied multivariate techniques. Wiley, ISBN: 978-0-471-31064-8.
Shultz, M.D., 2018. Two decades under the influence of the rule of five and the changing properties of approved oral drugs: miniperspective. *Journal of Medicinal Chemistry*, 62(4), 1701-1714.
Sivamaruthi, B.S., Kesika, P., Subasankari, K., Chaiyasut, C., 2018. Beneficial effects of anthocyanins against diabetes mellitus associated consequences-A mini review. *Asian Pacific Journal of Tropical Biomedicine*, 8(10), 471-477.
Sky-Peck, H.H., Thuvasethakul, P., 1977. Human pancreatic alpha-amylase. II. Effects of pH, substrate and ions on the activity of the enzyme. *Annals of Clinical & Laboratory Science*, 7(4), 310-317.
Szklarczyk, D., Santos, A., Von Mering, C., Jensen, L.J., Bork, P., Kuhn, M., 2016. STITCH 5: augmenting protein–chemical interaction networks with tissue and affinity data. *Nucleic Acids Research*, 44(D1), D380-D384.
Testa, B., Vistoli, G., Pedretti, A., van de Waterbeemd, H., Avdeef, A., Ivanciuc, O., Muresan, S., 2007. Molecular drug properties: measurement and prediction. Wiley, ISBN:9783527621286.
Tomasik, P., Horton, D., 2012. Enzymatic conversions of starch. *Advances in Carbohydrate Chemistry And Biochemistry*, 68, 59-436.
Trott, O., Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, 31(2), 455-461.
Tsuda, T., Ueno, Y., Aoki, H., Koda, T., Horio, F., Takahashi, N., Osawa, T., 2004. Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes. *Biochemical and Biophysical Research Communications*, 316(1), 149-157.
Tsuda, T., Ueno, Y., Kojo, H., Yoshikawa, T., Osawa, T., 2005. Gene expression profile of isolated rat adipocytes treated with anthocyanins. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1733(2-3), 137-147.
Tsuda, T., Ueno, Y., Yoshikawa, T., Kojo, H., Osawa, T., 2006. Microarray profiling of gene expression in human adipocytes in response to anthocyanins. *Biochemical Pharmacology*, 71(8), 1184-1197.
Veber, D.F., Johnson, S.R., Cheng, H.Y., Smith, B.R., Ward, K.W., Kopple, K.D., 2002. Molecular properties that influence the oral bioavailability of drug candidates. *Journal of Medicinal Chemistry*, 45(12), 2615-2623.
Vistoli, G., Pedretti, A., Testa, B., 2008. Assessing drug-likeness–what are we missing?. *Drug Discovery Today*, 13(7-8), 285-294.
Wallace, T.C., Giusti, M.M., 2015. Anthocyanins. *Advances in Nutrition*, 6(5), 620-622.
Wiernsperger, N., Rapin, J., 2010. Trace elements in glucometabolic disorders: an update. *Diabetology & Metabolic Syndrome*, 2(1), 1-9.
Yang, K., Bai, H., Ouyang, Q., Lai, L., Tang, C., 2008. Finding multiple target optimal intervention in disease-related molecular network. *Molecular Systems Biology*, 4(1), 228.
Zamora-Ros, R., Knaze, V., Luján-Barroso, L., Slimani, N., Romieu, I., Fedirko, V., González, C.A., 2011. Estimated dietary intakes of flavonols, flavanones and flavones in the European Prospective Investigation into Cancer and Nutrition (EPIC) 24 hour dietary recall cohort. *British Journal of Nutrition*, 106(12), 1915-1925.
Zhang, J., Sun, L., Dong, Y., Fang, Z., Nisar, T., Zhao, T., Guo, Y., 2019. Chemical compositions and α-glucosidase inhibitory effects of anthocyanidins from blueberry, blackcurrant and blue honeysuckle fruits. *Food Chemistry*, 299, 125102.
Zheng, Y., Ley, S.H., Hu, F.B., 2018. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nature Reviews Endocrinology*, 14(2), 88-98.

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