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IJSM is published 4 issues per year (March, June, September, December), and accepting manuscripts related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). Research areas covered in the journal are phytochemistry, biochemistry, biotechnology, ethnopharmacology, biological and pharmacological activities (antimicrobial activity, antioxidant activity, antiulcer activity, anti-convulsant activity, anti-anxiety activity, antidiabetic activity, anti-gout activity, antiprotozoal activity, anti-inflammatory activity, antispasmodic activity, antiparasitic activity, anti-mutagenic activity, anticholinesterase activity, antidepressant activity, hepatoprotective activity, anti-anxiety activity, anti-convulsant activity, anti-spasmolytic activity, anticancer activity). IJSM welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Authors are required to frame their research questions and discuss their results in terms of major questions in plant biology. Contribution is open to researchers of all nationalities. The following types of articles will be considered:

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Research Article

Effects of preharvest salicylic acid and oxalic acid treatments on blackberry (cv. Bursa 1) fruit quality

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Abstract: The aim of the current study was to determine how the pre-harvest different dosages of oxalic acid (OA) and salicylic acid (SA) affect the quality of blackberry (cv. Bursa 1) fruits at harvest. Thus, blackberry plants were sprayed with solutions containing 0.5 mM SA, 1 mM SA, 2.5 mM OA, and 5 mM OA seven and fourteen days to before the commercial maturity of fruits. Some characteristics of these fruits were investigated, including their biochemistry (phenolic compounds, organic acids, and general phytochemical characteristics), pomology (fruit width, fruit length, and fruit weight), and physiology (respiration). Regarding the results, the application of SA and OA increased fruit size and fruit weight by up to 40% and 23%, respectively, while leading to a reduction in soluble solid content by up to 7%. However, the organic acids and phenolic compounds with antioxidant impact were unaffected by this decline and were found to increase, especially with OA application. The control group's respiration rate was the highest among the harvested fruits, and the treatments lowered it by 30%. Consequently, the pre-harvest application of oxalic acid or salicylic acid could enhance the quality characteristics of blackberry fruit.

1. INTRODUCTION

Wild forms of blackberry (*Rubus fruticosus*), which belongs to the *Rubus* genusof the Rosaceae, are frequently encountered, especially in the Black Sea and transitional regions of Türkiye. This shows that Türkiye is among the centers of origin (Yilmaz *et al*., 2009). Despite this, Türkiye's blackberry culture is new compared to other major species (Onur, 1999). In fact, with a production of 3583 tons, our country is still far behind its blackberry production potential (TurkStat, 2023).

In contrast to the decreasing agricultural areas, there are changes in people's diets due to the continuous increase in the world population and the pandemic conditions we are in (King *et al*., 2020). In this context, blackberry, which belongs to the group of berries defined as functional foods, is a species with a wide range of uses that is integrated into many industry products and

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consumed fresh due to its aroma (Finn & Clark, 2012). Blackberries contain high levels of different biochemicals with high antioxidant effects, such as vitamins, minerals, polyphenols, and organic acids (Memete *et al*., 2023). This antioxidative effect is reported to reduce the risk of many chronic diseases, including microbial diseases, cancer, and cardiovascular diseases (Aglar *et al*., 2021; Martins *et al*., 2023). Since it is an early-fruiting and regular crop, a highly adaptable and labor-intensive agricultural branch, it is important as a complementary product in agricultural enterprises, especially regarding the utilization of women and child labor (Funt, 2017).

Despite the increasing cultivation and production of blackberries (FAO, 2022), optimization in their production has not yet been fully achieved, highlighting the need for practices that enhance both yield and quality (Finn and Clark, 2012). Consequently, pre-harvest practices aimed at improving fruit quality during harvest and ensuring post-harvest quality preservation, while taking pre-harvest factors into account, have recently gained significant importance (Batool *et al*., 2022; Garcia-Pastor *et al*., 2020; Hazarika & Marak, 2022; Martinez-Camacho *et al*., 2022; Onik *et al*., 2021;).Salicylic acid, also known as a plant growth regulator, and oxalic acid, an organic acid, regulate many different physiological events in plants (Hayat *et al*., 2013; Prasad & Shivay, 2017). In studies where these compounds are applied separately or together, it is stated that aging is delayed by suppressing ethylene production (Ansarifar, 2019), and weight and bioactive substance loss are reduced by reducing respiration and transpiration rates (Bal, 2019; Batool *et al*., 2022). They reduce the softening of fruits by reducing the activity of pectin methylesterase and polygalacturonase enzymes (Kumar *et al*., 2021). Fresh-cut fruits and vegetables inhibit polyphenol oxidase enzyme activity, keeping the product fresh longer (Liao *et al*., 2021). Increasing the synthesis of antioxidant-derived compounds increases tolerance to biotic and abiotic stress factors (Hayat *et al*., 2013; Prasad & Shivay, 2017). They ensure the maintenance of intracellular homeostasis (Kant *et al*., 2013).

This study investigated the effects of different doses of oxalic acid and salicylic acid, applied to plants before harvest, on the quality of blackberry fruits from the Bursa 1 cultivar harvested at eating maturity.

2. MATERIAL and METHODS

2.1. Plant Material

In this study, we used ‘Bursa 1’ blackberry plants cultivated in the experimental fields of Isparta University of Applied Sciences, Faculty of Agriculture, Isparta, Türkiye, in 2023. The plants were originally planted in 2015 with a row and intra-row spacing of 3.5 m × 1.2 m. The study site is located at an altitude of 1009 m, at coordinates 37° 50′ 13.6464” N and 30° 32′ 17.6316” E. The soil in the area is clayey-loam with moderate alkalinity and very high lime content. The organic matter and extractable P and Zn levels were low, the K content was medium, and the Mn, Fe, and Cu levels were sufficient, based on the criteria outlined by Jackson (1962) (see Table 1).

Table 1. Some physical and chemical characteristics of the experimental soil.

pH (1/2.5 water)	Structure	Lime (%)	Organic material	Nutrients that can be extracted (mg/kg)					
				P	K	Fe	Cu	Zn	Mn
8.1	Clayey-loam	26.0	1.9	12.6	124	3.1	0.9	0.38	2.9

2.2. Pre-Harvest Treatments of the Plant Materials

The plants were divided into five groups, with ten plants in each group. Whole above parts of plants were sprayed with an aqueous solution of salicylic acid (0.5 mM and 1 mM doses) or oxalic acid (2.5 mM and 5 mM doses) containing 0.01% Tween-20 to ensure that applied chemicals spread and adhered homogeneously as a thin film layer. Application concentrations

were determined by preliminary studies. Chemicals used are capable of breaking down easily in a short time. So, they do not have any drawbacks in terms of residue. Distilled water containing 0.01% Tween-20 was applied to the plants in the control group. The solution was sprayed (approximately 1 L per plant) with a hand sprayer on days 7 and 14 before commercial harvest (in order to set harvest time in advance, there were fruits on the plants at different ripening stages); harvesting was performed according to the color and taste (Eskimez *et al*., 2019). At the commercial ripeness stage, fruits of standard quality and size were harvested and transported to the laboratory without wasting any time.

2.3. Determination of the Pomological and Physiological Characteristics

2.3.1. *Fruit weight, length, and width*

Fruit weight was measured using an electronic balance (CPA 16001S; Sartorius, Göttingen, Germany), with an accuracy of 0.01 g. The width and length of the fruits were determined using a vernier caliper with a precision of 0.01 mm. The weight and size of the fruits were assessed based on 50 randomly selected fruits (Eskimez *et al*., 2019).

2.3.2. *Soluble solid content (SSC) and titratable acidity (TA)*

From each treatment group, 20 fruits on the day of analysis were squeezed with a juice extractor (Arzum AR1060, Istanbul, Turkey) and filtered with coarse filter paper to obtain juice samples, which were used for further analysis. SSC was measured using a digital refractometer (Atago Pocket PAL-1, Tokyo, Japan), and the results are presented as percentile values (Karaçalı, 2012). For determining TA, the fruit juice samples were titrated with 0.1 N sodium hydroxyl solution using phenolphthalein as an indicator. The results were expressed as malic acid %, and calculations were performed using the formula described by Karaçalı (2012).

2.3.3. *Respiration rate*

The fruits (100–125 g) were placed in a 500 mL glass jar, hermetically sealed, and incubated for 1 h at room temperature. Gas samples were collected by a gas-tight syringe after 1 h and injected. All measurements were made in split/splitless (S/SL) of the inlet in the split mode with a gas sampling valve with 1-mL gas sample using a fused silica capillary column (GS-GASPRO, 30 m x 0.32 mm ID., USA); a thermal conductivity detector (TCD) was used for respiration rate measurements.

2.3.4. *Extract preparation*

To prepare the fruit extracts for both HPLC and spectrophotometrical analyses, 10 g of fruit samples from each repetition were extracted with 10 mL of 80% acetone containing 0.2% formic acid, using a homogenizer for 2 min. Then, the samples were centrifuged (Eppendorf 5804R, NY, USA) at 20,000 *g* for 20 min at 4 °C (Selcuk and Erkan, 2016). Analyses were conducted when the last group was done. Extracts of previous groups were separated were stored at –20 °C till analyses.

2.3.5. *Spectrophotometric analysis*

2.3.5.1. Total Phenolic Content (TPC). The TPC was evaluated using the Folin-Ciocalteu technique as described and modified by Lola-Luz *et al*. (2014). The fruit juice was mixed with the Folin–Ciocalteu reagent and distilled water at a ratio of 1:1:18 (v/v/v) and left undisturbed for 8 min. Then, 7% sodium carbonate was added. After 2 h of incubation in the dark, the absorbance of the bluish solution was measured at 725 nm (Varian, Cary 100 Bio; Melbourne, Australia). Gallic acid was used as an external standard for plotting the calibration curve, and the results were expressed as gallic acid equivalent (GAE) of fruit juice (mg GAE 100 ml^{-1}).

2.3.5.2. Total Flavonoid Content (TFC). The aluminum chloride colorimetric method was used for determining the TFC, as described by Chang *et al*. (2002). Briefly, 50 µL of juice was collected in 10 mL tubes and mixed with 950 µL of methanol and 4 mL of distilled water. Then,

300 µL of sodium nitrite solution (5% in water) was added to the mixture. Then, 300 µL aluminum chloride solution (10% in water) was added and the mixture was left undisturbed for 5 min. Next, 2 mL of sodium hydroxide solution (1 M, in water) was added, and the total volume of the mixture was made up to 10 mL by adding distilled water. After incubating the mixture for 15 min, spectrophotometric analyses were conducted at 510 nm. The TFC was calculated from the quercetin calibration curve, and the results were expressed as mg quercetin equivalent per liter.

2.3.5.3. DPPH Assay. Antioxidant activity analyses were performed using the DPPH method. First, 50% inhibition concentration (IC_{50}) was calculated by evaluating percentage inhibition against the sample concentration. Then, concentrations up to the IC_{50} value of the samples were taken and the ability to remove DPPH radicals was determined using the method described by Mertoğlu *et al*. (2022). All results were expressed as percentages (%) in which ascorbic acid was used as an indicator.

2.3.6. *Quantification of organic acids and phenolic compounds by HPLC-UV*

Samples were first shaken for 1 h and centrifuged at 14,000 rpm for 15 min. Then, the supernatant was filtered using a 0.45 µm membrane filter. The filtered juice was analyzed by an Agilent 1260 HPLC device (Agilent Technologies, CA, USA) equipped with the Chemstation software, a quaternary pump, an autosampler, and a UV detector.

The organic acids were determined using an ACE-C18 column (4 mm × 150 mm, 5 µm; Hichrom Ltd., Theale, UK). The mobile phase consisted of a 10 mM aqueous solution of potassium phosphate (pH 2.2 with *ortho*-phosphoric acid) with a flow rate of 1 mL min^{-1}. The injection volume was 20 µL and the detector was set to 245 nm for ascorbic acid and 210 nm for all other organic acids (Fu *et al*., 2015).

An ACE-C18 (4.6 mm × 150 mm, 5 µm; Hichrom Ltd., Theale, UK) column was used for the chromatographic separation of phenolic compounds. Details of chromatographic conditions are given in Table 2.

Table 2. Chromatographic conditions of HPLC method.

Parameters	Conditions	
Mobile phase	A: Ultrapure water containing 0.1% acetic acid. B: Acetonitrile containing 0.1% acetic acid.	
Mobile phase flow rate	1.0 mL min^{-1}	
Column	ACE-C18 (4.6 mm × 150 mm, 5 µm)	
Column temperature	25 °C.	
Injection volume	10 µL	
Run time	30 min.	
Detection wavelengths	280 nm for syringic acid, protocatechic acid, and gallic acid. 225 nm for vanillic acid. 305 nm for p-coumaric acid. 330 nm for caffeic acid and chlorogenic acid.	
Elution	Gradient	
	Time min.	B% (Volume)
	0.00	8
	3.25	10
	8.00	12
	15.00	25
	15.80	30
	25.00	90
	25.40	100
	30.00	100

The mobile phase flow rate was maintained at 1.0 mL min^{-1}. Mobile phase A was ultrapure water containing 0.1% acetic acid, whereas mobile phase B was acetonitrile containing 0.1% acetic acid. The gradient conditions were as follows: 0–3.25 min, 8–10% B; 3.25–8 min, 10–12% B; 8–15 min, 12–25% B; 15–15.8 min, 25–30% B; 15.8–25 min, 30–90% B; 25–25.4 min, 90–100% B; 25.4–30 min, 100% B. The injection volume was 10 µL and the column temperature was maintained at 25 °C. The detection wavelengths were selected based on the wavelengths at which the phenolic compounds to be analyzed had maximum absorption. Syringic acid, protocatechuic acid, ferulic acid, ellagic acid, quercetin icand gallic acid were detected at 280 nm. Caffeic acid was detected at 330 nm while *p*-coumaric acid was detected at 305 nm (Wen *et al*., 2005).

2.4. Statistical Analysis

The trees in field were divided to five repetitions and each repetition consisted of ten plants according to randomized parcel design. The data were evaluated by the analysis of variance (ANOVA) using one-way ANOVA in the statistical analysis software Minitab-17. Significant differences (at $p < 0.05$) among treatments were determined using Tukey's multiple comparison test (Düzgüneş *et al*., 1987).

3. RESULTS and DISCUSSION

3.1. Fruit Weight, Fruit Length and Fruit Width

The effects of SA and OA treatments on fruit weight, fruit length, and fruit width of Bursa 1 blackberry cultivar at harvest were found to be statistically significant ($p<0.05$), and the results are presented in Figure 1. When fruit weight, length and width were considered, higher values were obtained in all treatments compared to the control. The highest fruit weight (18.2 g) was found in SA 1 mM treatment, followed by OA 5 mM (15.5 g), SA 0.5 mM (15.3 g), OA 2.5 mM (14.9 g), and control (14.8 g). Fruit size has a strong positive relationship with fruit weight and is the most important trait affecting weight along with volume (Çolak *et al*., 2022; Yaman, 2022). The results of the study were in this direction, and the longest (23.5 mm) and widest (3.1 mm) fruits were measured in 1 mM SA treatment, where the highest fruit weight was obtained. OA was in the same statistical group as the control regarding these characteristics. When pre-harvest SA treatment was applied to plum trees, the fruit weight of the treated trees was found to be around 25% higher than that of the controls (Martinez-Espla *et al*., 2019). In another study conducted on cherries, the application of pre-harvest SA resulted in an increase in fruit weight between 13% and 37% (Gimenez *et al*., 2014). This improvement in pomological traits is thought to be since salicylic acid is mainly involved in the processes of cell division and expansion.

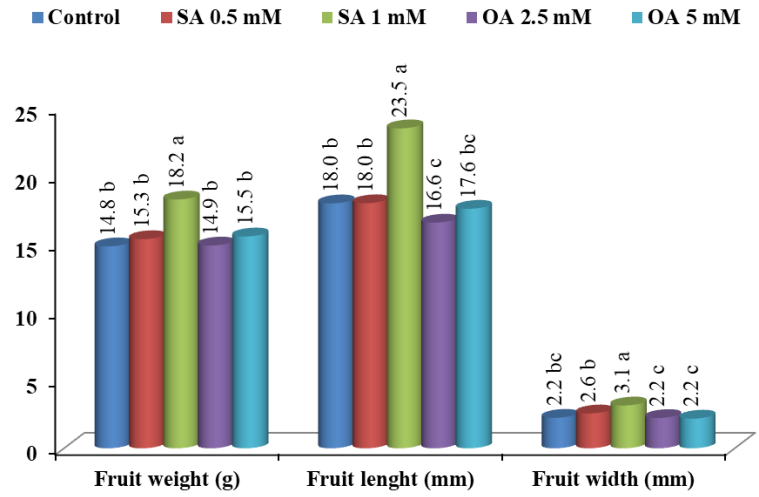


Figure 1. Effects of pre-harvest SA and OA treatments on fruit weight, fruit width and fruit length at harvest of Bursa 1 blackberry cultivar.

3.2. Soluble Solid Content, Titratable Acidity and Respiration Rate

The ripeness and flavor of horticultural crops are shaped by the composition of sugars and organic acids and the ratio of their sum to each other (Obenland *et al*., 2011). Therefore, the accumulation and conservation of these molecules is very important. In the study, the SCC value was lower than the control in the fruits obtained from all treatment groups (see Figure 2). The highest SSC (16.2%) was measured in the control and distinguieshed statistically from 5 mM OA (15.6%), 2.5 mM OA (15.5%), 0.5 mM SA (15.1%) and 1 mM SA (15.1%) treatments (p<0.05). This is thought to be mostly caused by the treatments' effects on fruit size, as larger fruits have more intercellular space and less dry matter accumulation per unit area (Çolak *et al*., 2022).

Analysis of the blackberry respiration rate at harvest regarding groups shown in Figure 2 revealed that, differences between the groups were significant and the order was as follows; control (4.0 mL CO_2 $kg^{-1}h^{-1}$)> five mM OA (3.3 mL CO_2 $kg^{-1}h^{-1}$)> 2.5 mM OA (3.2 mL CO_2 $kg^{-1}h^{-1}$)> one mM SA (3.1 mL CO_2 $kg^{-1}h^{-1}$)> 0.5 mM SA (2.8 mL CO_2 $kg^{-1}h^{-1}$). It has been previously reported that application of SA and OA slow down metabolic activity and downregulate the *ACO1* and *ACS2* genes responsible for ethylene biosynthesis, which are involved in ethylene production, resulting in late harvest (Kumar *et al*., 2021). Thus, SA indicating potential for post-harvest use as well. As seen in Figure 2 pre-harvest SA treatment reduced respiration up to 30% at harvest. A grape study's findings highlighted how pre-harvest applied SA could effectively effectively lowering weight loss and bioactive compund loss by lowering transpiration and respiration (Sabır & Sabır, 2017).

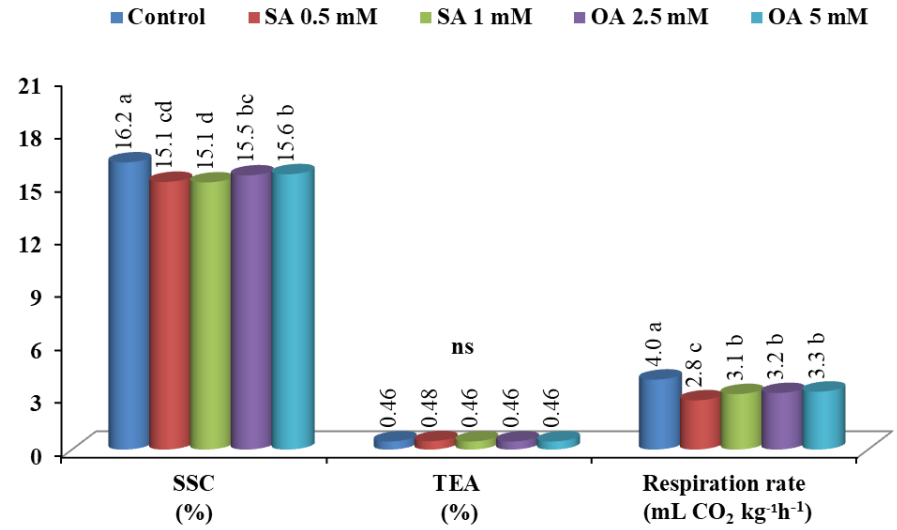


Figure 2. Effects of pre-harvest SA and OA treatments on water-soluble dry matter, titratable acidity and respiration rate of Bursa 1 blackberry cultivar at harvest.

In addition to their many health benefits, fruits and vegetables are a good source of natural antioxidants, which offer protection against harmful reactive oxygen species (ROS) and are linked to lower incidence and mortality rates of degenerative diseases like cancer and cardiovascular disease (Robinson *et al*., 2020). Because of their effects on low density lipoproteins, phytochemicals like phenolics, flavonoids, and anthocyanins-all of which are generated by the phenylpropanoid pathway have been linked to a decreased risk of heart disease by the ability to scavenge reactive oxygen species (ROS) (Yang & Kortesniemi, 2015). They are also significant because they add to the color, astringency, bitterness, and flavor of fruits and vegetables, which are characteristics of high nutritional quality (Xu *et al*., 2023).

Figure 3 illustrates how preharvest SA and OA treatments affected the total phenolic content of blackberries at harvest. Treatments had statistically significant effects on the TPC at harvest (p<0.05). TPC of the OA and SA treatments was higher than that of the control. Among the

treatments OA-treated blackberries had higher TPC than SA-treated blackberries. The 5 mM OA treatment had the highest total phenolic content (730.6 mg GAE 100mL^{-1}), which was followed by the 2.5 mM OA (706.1 mg GAE 100mL^{-1}), 1 mM SA (697.0 mg GAE 100mL^{-1}), 0.5 mM SA (683.3 mg GAE 100mL), and control (683.1 mg GAE 100mL^{-1}).

The highest total phenolic content (730.6 mg GAE 100mL^{-1}) was measured in 5 mM OA treatment, followed by 2.5 mM OA (706.1 mg GAE 100mL^{-1}), one mM SA (697.0 mg GAE 100mL^{-1}), 0.5 mM SA (683.3 mg GAE 100mL^{-1}) and control treatment (683.1 mg GAE 100mL^{-1}). Many species such as mango (Zheng *et al*., 2012), banana (Huang *et al*., 2013) and cherry (Martínez-Esplá *et al*., 2014) have shown evidence of an increase in the TPC at harvest when OA was applied prior to harvest; however further research was needed to determine the mechanism underlying this effect (Serna-Escolano *et al*., 2021). Activation of phenylalanine ammonia-lyase (PAL) activity, a crucial enzyme in the phenylpropanoid pathway involved in phenolic production, may be the cause of the effect of OA treatment on increasing phenolic (Martínez-Esplá *et al*. ,2019). Likewise Mirdehghan *et al*. (2012) SA may boost the activity of phenylalanine ammonia lyase, an enzyme involved in the synthesis of flavonoids and phenolics via the phenylpropanoid pathway, and hence increase the quantity of phenolic compounds.

The effect of OA and SA treatments on the total flavonoid content of blackberries is shown in Figure 3, and the effect of treatments was found to be statistically significant (p<0.05). Fruits treated with 0.5 mM (587.3 mg CE L^{-1}) of SA and 5 mM (600.7 mg CE L^{-1}) of OA had a lower TPC, while highest concentration of flavonoids were measured from 2.5 mM OA (665.9 mg CE L^{-1}) and 1 mM SA (644.1 mg CE L^{-1}). According to reports, OA and SA functions as a natural antioxidants by decreasing ascorbic acid oxidation and inhibiting lipid peroxidation in vitro in a concentration-dependent manner (Batool *et al*., 2022; Gacnik *et al*., 2021). A crucial enzyme in the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL) catalyzes the conversion of phenylalanine to trans-cinnamic acid. Shikimic acid route, the primary metabolism, and the phenylpropanoid pathway, the secondary metabolism, are linked by the PAL (Dixon & Paiva, 1995). These findings lead us to hypothesize that activation of PAL enzyme activity may be the cause of the greater total phenol and flavonoid concentrations in blackberry fruits treated with OA and SA at harvest.

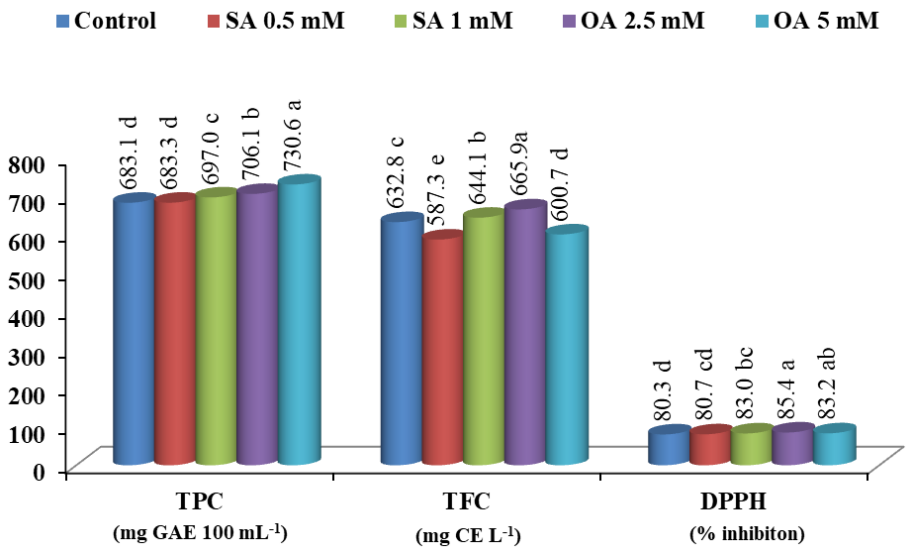


Figure 3. Effects of pre-harvest SA and OA treatments on total phenolic content, total flavonoid content and DPPH values.

Antioxidant enzymes, together with antioxidant compounds (phenolics, flavonoids, etc.), play a role in scavenging free radicals and ROS formed during the development of crops (El-Zaeddi *et al*., 2017). An increase in the antioxidant capacity of products has also been reported with pre- or post-harvest applications (Sayyari *et al*., 2011; Valero *et al*., 2011). In the present study,

the antioxidant activities of blackberries at harvest were slightly increased by pre-harvest SA and OA treatments. Similar to the total phenolic and total flavonoid contents, the lowest antioxidant activity (80.3%) was measured in the control treatment, while the highest values were measured in the OA treatment (see Figure 3). Similar to the results of this study, higher antioxidant activity at harvest in OA-treated fruits was also reported in studies conducted in different species (El-Zaeddi *et al*., 2017; Martinez-Espla *et al*., 2014).

Phenolic compounds are extremely important in terms of their positive effects on health and their regulation of important physiological events. The variation of the phenolic compounds analyzed according to the treatments showed significant differences between the treatments, as shown in the Figure 4. Among the phenolic compounds examined, syringic acid, which is prominent in terms of quantity, had the highest value in the control group (21.44 mg L^{-1}) and the lowest value (16.74 mg L^{-1}) in the group treated with one mM SA (see Figure 4). A similar situation was observed in other phenolic compounds, and in general, a decrease in phenolic compounds was observed as a result of the treatments compared to the control. This is thought to be mainly due to the increase in fruit weight in parallel with the treatments. As it is known, the increase in cell size leads to an increase in intercellular space (Çolak *et al*., 2022). This causes a decrease in the amount of biochemicals produced per unit area. There are studies on the negative correlation between pomological and chemical properties of fruit in different species (Yaman, 2022). It is also known that SA and OA delay phytochemical accumulation by suppressing the effect of ethylene and abscisic acid (Ansarifar, 2019; Kumar *et al*., 2021).

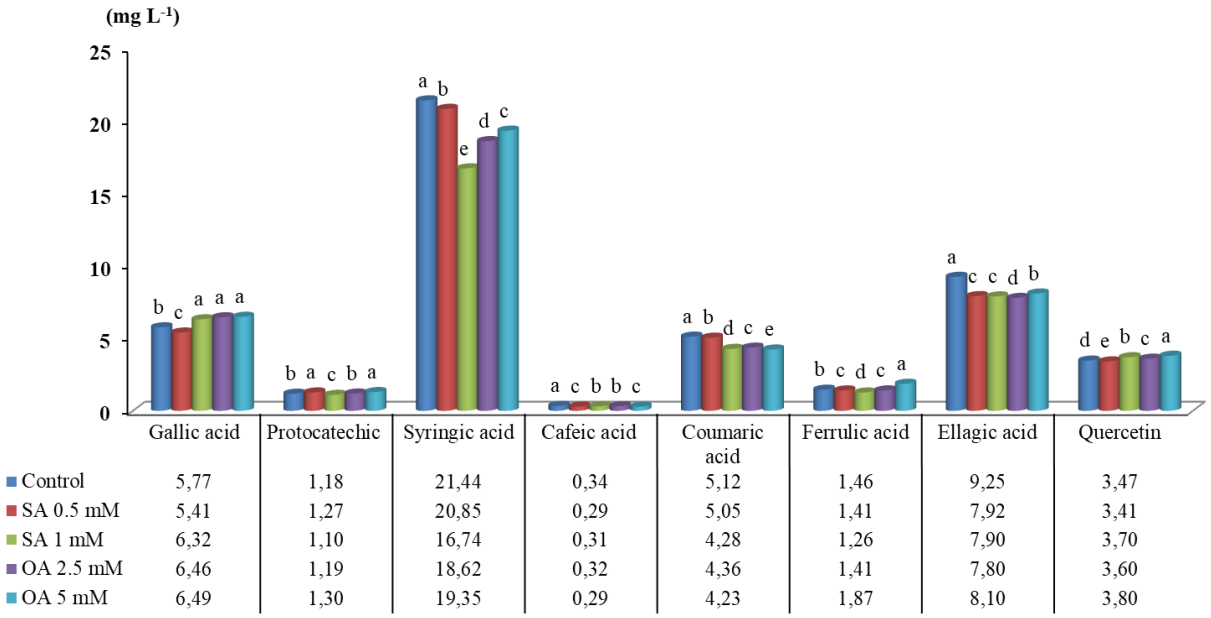
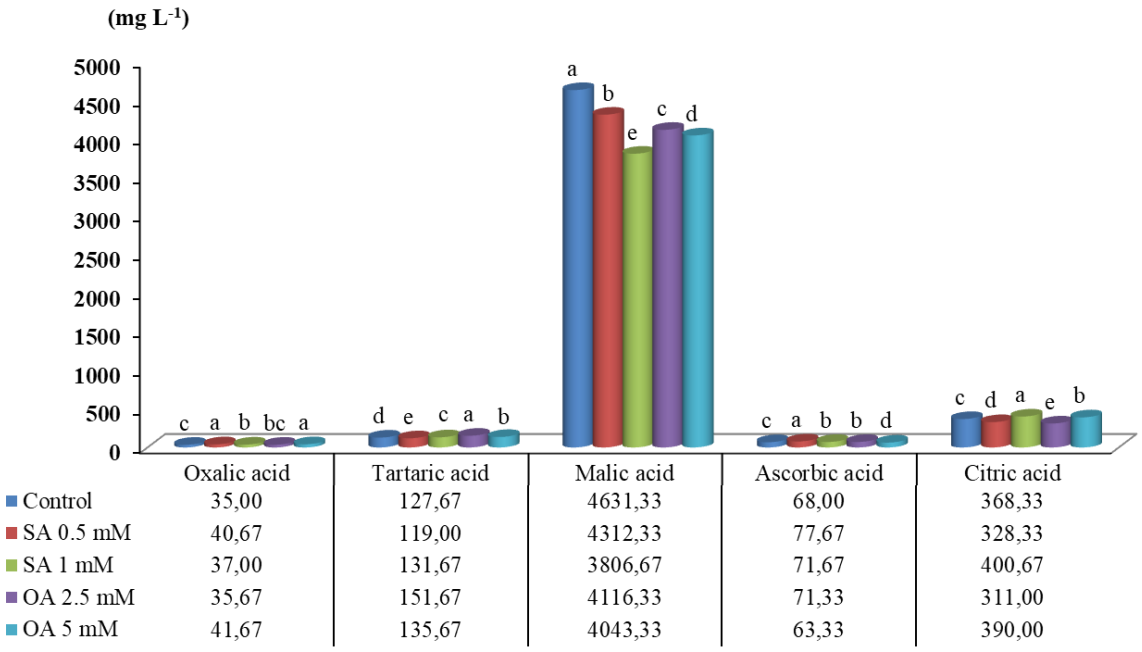


Figure 4. Effects of pre-harvest SA and OA applications on some phenolic compounds.

The organic acids examined in the study are given in Figure 5, and the effects of the treatments on all organic acids were found to be significant. Malic acid was determined as the dominant organic acid. The finding is in line with the study conducted by Kafkas *et al*. (2006), while Mikulic-Petkovsek *et al*. (2017) reported that citric acid was found to be the dominant acid of the same variety. On the other hand, it was observed that the dominant acid may vary under different factors in blackberry species (Mikulic-Petkovsek, 2021). The order of the organic acids analyzed was Malic acid> citric acid> tartaric acid> ascorbic acid> oxalic acid (see Figure 5).

Taste, which is the main reason for consumer preference, is one of the criteria shaped by organic acids, and the main reason for taste loss is the breakdown of organic acids (Obenland *et al*., 2011). In addition, organic acids are the main components that provide a low-pH environment in fruits and limit the activity of harmful microorganisms that cause spoilage (Adamczak *et al*.,

2019). The effects of the applied chemicals on organic acids showed differences. While the control stood out in terms of the dominant organic acid, oxalic and tartaric acid accumulation increased with oxalic acid applications. Salicylic acid stimulated the accumulation of ascorbic acid and citric acid. Although similar results were obtained in all groups in terms of cumulative acid accumulation, fruit size and weight were higher in the treated groups. Therefore, it can be said that oxalic and salicylic acid treatments contributed to organic acid accumulation. SA and OA contribute positively to the accumulation of organic acids by increasing citrate synthase and NAD-malate dehydrogenase enzyme activities that synthesize organic acids (Brizzolara *et al*., 2020).



	Oxalic acid	Tartaric acid	Malic acid	Ascorbic acid	Citric acid
Control	35,00	127,67	4631,33	68,00	368,33
SA 0.5 mM	40,67	119,00	4312,33	77,67	328,33
SA 1 mM	37,00	131,67	3806,67	71,67	400,67
OA 2.5 mM	35,67	151,67	4116,33	71,33	311,00
OA 5 mM	41,67	135,67	4043,33	63,33	390,00

Figure 5. Effects of pre-harvest SA and OA applications on some organic acids.

4. CONCLUSION

In the current study how the preharvest application of SA and OA effect some quality characteristics of blackberry fruits were investigated. Regarding the results, applied OA and SA doses had positive effects on the pomological, biochemical and physiological characteristics examined at harvest. It was determined that SA applications (especially 1 mM SA) decreased the respiration rate at harvest, decreased the amount of SCC by increasing the fruit weight. On the other hand, OA applications were found to be more effective in improving biochemical characteristics as well as quality characteristics. As a result, it was observed that OA and SA applied before harvest could contribute to the improvement of quality characteristics and biochemical contents of Bursa 1 blackberry cultivar at harvest. However, the doses and timing of application of these substances may vary among species and even cultivars, and further research on this subject is thought to be necessary.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Kerem Mertoğlu: Conceptualization, investigation, methodology, software, writing – original draft, review and editing. **İlknur Eskimez:** Conceptualization, investigation, methodology, software, writing – original draft, review and editing. **Mehmet Polat:** Resources and editing.

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Research Article

Analysis of accessible and total phosphate contents in different foods, to assess their suitability for use by dialysis patients

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Abstract: There is a direct link between hyperphosphatemia and increased mortality in end-stage kidney disease, which can be attributed to the higher consumption of ultra-processed foods. Thus, the study aimed to analyze the total phosphorus content and relative amount of available phosphorus in various manufactured processed foods (MPF) and home processed foods (HPF), specifically for use by dialysis patients. The results of the total phosphorus content were found in the range of (26 to 290 mg P/100g). The highest concentration (290 mg/100g) was found in nan bread (HPF), followed by boiled eggs (255mg/100g), chicken (192mg/100g) and beans (91mg/100g). For MPF samples, the chicken (195mg/100g), whole meal bread (193mg/100g), and wheat and white bread wraps for both Asda and Tesco (99, 124 mg/100 respectively) respectively exhibited high phosphorus content. In this study, relative available P concentrations were in the range of (5-42.5 mg P/100g) for MPF and (6-38 mg P/100g) for HPF. While the highest concentration (42.5 mg/100g) was found in chicken (MPF), followed by whole meal bread, and Coca-Cola. For HPF Nan bread (37.8 mg P/100g) showed high content followed by boiled eggs and Chicken. HPF generally exhibited higher levels of both total phosphorus content and extractable phosphorus compared to manufactured processed foods (MPF). This is an important consideration for patients, particularly those with kidney disease, as they should be mindful of their phosphorus intake when consuming HPF.

1. INTRODUCTION

Phosphorus is one of the most abundant minerals in the crustal rock on the Earth. It is found widely in nature and roughly exists in nearly all foods (Metson *et al.*, 2016). Phosphorus is an essential mineral that is necessary for a well-balanced diet, as it plays a critical role in bone composition and the regulation of numerous physiological processes within the body (Hifizah, 2011). In an individual with normal renal function, the kidney has a crucial role in regulating phosphorus and calcium homeostasis by excreting excess phosphorus. When kidney function deteriorates, the phosphorus levels increase in the blood, which is common in patients with chronic kidney disease (Kestenbaum *et al.*, 2005; González-Parra *et al.*, 2012). Despite the increase in the prevalence of the condition in many countries, chronic kidney diseases (CKD) remain a problem in the world. For example, CKD is a widespread clinical condition that affects

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over 800 million individuals suffering from various kidney disorders (Thongprayoon *et al*., 2021) and affects more than 20 million American people (Kovesdy *et al*., 2006). According to the Global Burden of Disease research, there were 697.5 million cases of CKD worldwide, representing a 9.1% prevalence rate (Okpechi *et al*., 2022). Phosphorus additives in food have been reported" to be the main cause of increased serum phosphate levels. Processed foods with food additives contain 70% more phosphorus than similar foods without food additives (Cupisti *et al*., 2004). Thus, manufactured meals may contain higher levels of phosphorus compared to naturally available foods, and the body may absorb this phosphorus more readily (Noori *et al*., 2010). However, a crossover experimental design on 8 patients with chronic kidney disease was conducted to compare the effect of meat-based and vegetarian diets on serum phosphate levels, with both diets containing equivalent concentrations of protein and phosphorus. The results revealed that over the course of seven days, participants following the vegetarian diet exhibited significantly lower blood serum phosphate levels compared to those that followed the meat-based diet (Moe *et al*., 2011). This can be attributed to the greater bioavailability and absorption of phosphorus from animal sources compared to that from plant sources (Noori *et al*., 2010). It has been illustrated that after three months, a significant reduction in blood serum phosphate levels (0.6 mg/day) was observed in dialysis patients who were advised to restrict their intake of food additives containing phosphorus, such as certain beverages and processed meats (Sullivan *et al*., 2009). In patients with renal failure, it is a crucial aspect of effective therapeutic management to restrict phosphorus intake, particularly after the initiation of dialysis (Calvo & Uribarri, 2021). To understand the effect of phosphorus on the patient with end-stage CKD, it is essential to gather data regarding the phosphorus content in commonly consumed ready-made meals. Typically, the Western diet provides phosphorus intake ranging from 1000 to 2000 mg per day. However, the recommended daily phosphorus intake for patients with advanced-stage chronic kidney disease (CKD) is considerably lower, ranging from 550 to 1100 mg per day. Therefore, the bioavailability of phosphorus in the diet is generally about 60% (Kooienga, 2007).

Numerous pre-clinical and clinical investigations report that high levels of phosphate can adversely affect health, both in healthy individuals and in patients with various conditions such as cardiovascular disease, bone disorders, or kidney disease. Therefore, the recommended dietary allowance (RDA) for phosphorus is approximately 700 mg per day for adults; however, the majority of individuals consume significantly higher levels of phosphate, nearly double the RDA (Erem & Razzaque, 2018). Thus, the main objective of the present study is to investigate the phosphorus content in both MPF and HPF, as well as to estimate the relative available phosphorus content in these foods and their relationships.

2. MATERIAL and METHODS

2.1. Food Samples

The samples tested in this study included manufactured processed foods (MPF) and home-processed foods (HPF). The MPF samples were acquired from a local market in the United Kingdom, while the HPF samples were prepared using local methods at home. The daily diet of a typical individual consuming manufactured processed foods (MPF) was selected as the study sample, comprising breakfast items such as yogurt, wheat and white bread, and milk; lunch consisting of fried potatoes and Coca-Cola; and dinner featuring basmati rice, chicken breast, baked beans in tomato sauce, and whole meal bread. Additionally, the normal mixed diet for an individual for one day, incorporating both home-processed foods (HPF) and MPF, included breakfast with yogurt, naan bread (from local shops), milk, and eggs; lunch with fried potatoes and Coca-Cola; and dinner of basmati rice, chicken breast, and baked beans in tomato sauce.

A detailed list of the manufactured processed food (MPF) samples used in this study, along with their suppliers, includes items such as whole milk and Mission Deli Wheat and White Wraps (8-pack), both obtained from Tesco. White tortilla bread, Rice, Chicken tikka & basmati

rice (weight watcher), and soft drinks (Coca-Cola) were gathered from Asda. Tasty whole meal bread (KINGSNILL), fried potato chips, ready-cooked sliced Chicken breast, yoghurt (Onken) Natural set, beans, baked, in tomato sauce, canned (Beanz) were from Iceland. Naan bread was obtained from local shops.

2.2. Chemicals

All chemicals used in this study include hydrochloric acid, approx. 36% w/w HCl (Fisher chemical), ammonium molybdate tetrahydrate (Santa Cruz), ammonium metavanadate (Santa Cruz), potassium dihydrogen phosphate, KH2PO4 (BDH Laboratory), toluene (Fisher chemical), pepsin from porcine gastric mucosa (SIGMA-ALDRICH), pancreatin (P-1750, from porcine pancreas) (SIGMA-ALDRICH), bile extract porcine (SIGMA-ALDRICH), salivary alpha-amylase from human saliva (SIGMA-ALDRICH), sodium bicarbonate (BDH Laboratory), NaHCO3 (BDH Laboratory).

2.3. Analytical Methods

In this study, MPF samples were sourced from three markets: Asda, Tesco, and Iceland. Home-processed foods (HPF) were prepared using similar methods as the methods used for the MPF samples, but in a home setting. Both the MPF and HPF foods were kept cooled in the laboratory conditions to avoid contamination. All glassware was acid-washed in a laboratory dishwasher, rinsed with distilled water and dried before use. However, deionized iron free water was used during the experiment for the dilution of samples.

2.4. Total Phosphorus Determination

2.4.1. *The preparation of sample solution by dry combustion*

All samples were prepared based on the procedures described by Nielsen (Nielsen, 2017) after a few modifications. Samples of MPF including whole milk, white tortillas (from Asda), wheat and white bread (from Tesco), basmati rice, Coca-Cola, whole meal bread, fried potatoes, chicken breast, yogurt, and beans baked in tomato sauce, along with seven types of home-processed foods (HPF) such as basmati rice, fried potatoes, chicken breast, naan bread, yogurt, beans baked in tomato sauce, and eggs, were collected and transported to the laboratory. One gram of the food sample was weighed and placed into a crucible, which was then positioned in a cool muffle furnace. The temperature was gradually increased to 450 °C and maintained at this level overnight until only a whitish-grey ash remained. After the muffle furnace was turned off, the crucible was rapidly transferred to a desiccator to cool before it was weighed.

Ash content was determined by using this equation: [(W5 – W4)/W3] *100

Where W3: the weight of the sample, W4: the weight of the sample and weight of the crucible after dry combustion, W5: the weight of the sample and crucible before dry combustion.

Subsequently, the crucible was placed in a water bath, and 5 mL of approximately 6 M hydrochloric acid was added, followed by the evaporation of the solution to dryness. After that, the residue was moistened with 1 mL of approx. 36% hydrochloric acid, and lightly evaporated to dryness. After approximately 5 mL of water was added, and the solution was evaporated once more. Finally, the residue was collected, transferred to a 25 mL volumetric flask, filtered using Whatman filter paper, and then diluted to a final volume of 25 mL.

2.4.2. *Analysis of sample solution*

In the spectrophotometric procedure for total phosphorus determination using a Libra S12-Biochrom (UK) spectrophotometer, various sample volumes (3 mL, 5 mL, and 10 mL) were transferred into a 50 mL volumetric flask, depending on the type of food. Following this, 5 mL of approximately 5 M hydrochloric acid and 5 mL of ammonium molybdate were added. Ammonium metavanadate reagent was ten added and the solution was filled up to the mark. The solution was allowed to stand for 30 minutes to ensure the development of the yellow color, after which the absorbance was measured against a blank at 400 nm

The calibration curve was employed to determine the phosphorus concentration (y) in the working standard solution. This curve was generated at a detection wavelength of 400 nm, using a series of working standard solutions with concentrations ranging from 0 to 50 µg/mL. The calibration curve was then applied to calculate the phosphorus concentration in each sample, following the equation below:

$$C1V1=C2V2 \ \mu g/mL,$$

2.5. In vitro Model

In vitro enzyme digestion procedure was performed according to the procedure of Miller (Miller *et al*., 1981), with a few modifications in the preparation of reagent, enzymes and digestion process to adapt methods with the equipment and glassware that were available in the laboratory.

2.5.1. *Preparation of sample*

Samples of MPF (whole milk, white tortillas bread (Asda), wheat & white bread (Tesco), basmati rice, coca cola, whole meal bread, fried potatoes, chicken breast, yoghurt, beans baked in tomato sauce) and HPF (basmati rice, fried potatoes, chicken breast, Nan bread, yoghurt, beans baked in tomato sauce and eggs) were used in these experiments to estimate relative available phosphorus content. The samples were transported to the laboratory, where 20 g of each was weighed and transferred into 100 mL beakers to initiate the digestion process.

2.5.2. *Preparation of reagent and enzymes*

A pepsin solution was prepared by dissolving 16 g pepsin (from porcine gastric mucosa) in 100 mL of 0.1 M HCl (Miller *et al*., 1981). A Pancreatin-bile extract mixture was prepared by weighing 4 g pancreatin (P-1750, from porcine pancreas) and 25 g of porcine bile extract, then dissolving the mixture in 1 L of 0.1 M $NaHCO_3$, as described by Miller *et al*. (1981) α-amylase: 20mL of 0.85% saline solution (sodium bicarbonate) was added to a bottle of Salivary alpha-amylase from human saliva 1KU to obtain α-amylase 50 KU.

2.5.3. *In vitro digestion methods*

For both MPF and HPF samples (20 g), 15 mL of deionized water was added to 100 mL beakers and the mixture was stirred using a glass rod. For the following food samples: white tortilla bread (Asda), wheat & white bread (Tesco), naan bread, basmati rice, whole meal bread, fried potatoes, and beans baked in tomato sauce, 0.125 mL of salivary amylase was added. The mixture was shaken and then incubated at 37°C for 5 minutes at a shaking speed of 95 rpm. This step aimed to digest the starch in these foods, which are known to have a high starch content. The pH of all samples was first adjusted to 2.0 by adding 6 M HCl. Subsequently, 1.5 mL of pepsin was introduced to each sample, and they were incubated in a shaking water bath at 37°C for 2 hours. After the incubation period, the pH was readjusted to below 7.0 by adding 1 M $NaHCO_3$. A 2.5 mL portion of the pancreatin-bile extract mixture was added to the samples, which were then incubated again in a shaking water bath at 37°C for 2 hours. At the end of the incubation period, the digested tubes were removed and centrifuged at 4000 rpm for 40 minutes, and then 2 mL of supernatant was removed into Eppendorf tubes for more centrifuges. After the centrifuge, digested samples were removed for estimation of the relative available phosphorus content. 0.1mL of samples was transferred into a 50 mL volumetric flask followed by the addition of 5 mL of approximately 5M hydrochloric acid and 5 mL of ammonium molybdate – ammonium metavanadate reagent and filled up to the mark with water. The mixture was allowed to stand for 30 minutes to ensure color development, after which the absorbance was measured against a blank at 400 nm.

2.6. Conversation

The phosphorus values can be converted to phosphate by using the following equation:

Phosphate (mg/100g) = phosphorus (mg/100g) *3.06

Find out this equation by using the molecular weight (MW) of PO4:

Where: P (MW) =31, O (MW) =16, and then PO4 (4*16) +31 = 95

It means that every 95 phosphate contains only 31 phosphorus. However, the µg/mL values can be converted to mg/mL dividing by 1000.

2.7. Percentage of Extractable Phosphorus in Food Samples

The extractable phosphorus value can be converted to a percentage by using this equation:

$$\text{In vitro extractable P \%} = (C1/C2) * 100.$$

Where: C1 = concentration of relative available phosphorus; C2 = concentration of total phosphorus.

2.8. Statistical analysis

In this study, each sample was repeated three times and values are given as mean + standard deviation. For both methods, Data was entered into Microsoft Excel, and the IBS SPSS software program 2010 (version 20, USA) was utilized to conduct a normality test using the Shapiro-Wilk test to assess the normality of the data. The data were normally distributed; therefore, One-Way ANOVA and Independent Sample *t*-test at confidence interval for mean 95% were used to determine significant differences between samples.

3. FINDINGS

3.1. Total Phosphorus Content

The results of the total phosphorus content for all food samples showed different variances between samples as illustrated in Table 1, Figure 1a. It can be observed that the phosphorus concentrations in the food samples ranged from 26 to 290 mg P/100 g of fresh samples. The lowest concentration, 26 mg/100 g, was found in Coca-Cola, while the highest concentration of 290 mg/100 g was observed in naan bread, which was followed by boiled eggs (255 mg/100 g), chicken (192 mg/100 g), and beans (91 mg/100 g). In the MPF samples, chicken (195 mg/100 g), whole meal bread (193 mg/100 g), and wheat & white bread wraps from both Asda and Tesco (99 and 124 mg/100 g, respectively) exhibited high phosphorus content. The results indicated a significant difference in the total phosphorus content between the bean and rice samples ($p < 0.05$). Furthermore, the analysis revealed that the effects of manufactured processed foods did not statistically influence the total phosphorus content in chicken, potatoes, and yogurt. Ash content represents the amount of ash material that remained in fresh food samples after putting them in a muffle furnace overnight. All food samples exhibited varying levels of ash content. Generally, phosphorus is well recognized as an essential mineral in the human body. As a result, phosphorus is abundantly found in both plant and animal food sources, including meat, poultry, fish, eggs, and dairy products, as well as in nuts and legumes. It is widely available as a food additive in processed foods such as meats, soft drinks and bakery goods (Willett, 2008). There is a scarcity of research on the determination of total phosphorus and the estimation of in vitro extractable phosphorus across a diverse range of food samples. The difference in food and technological processes account for the observed variation in phosphorus content. When compared to other data, the results obtained are generally consistent with those published by Esperance (2018). For instance, the phosphorus concentrations in whole milk, basmati rice, and Coca-Cola were reported to be 83 mg, 33 mg, and 16 mg per 100 g of fresh sample, respectively. In contrast, our results indicated concentrations of 69 mg, 30–38 mg, and 26 mg per 100 g, respectively. Research indicates that food additives are likely the most significant factor influencing total phosphorus content. This is primarily due to the fact that certain products, such as chicken and meat, are often enhanced with phosphate additives, which can elevate their total phosphorus content (Kalantar-Zadeh *et al*., 2010). Nonetheless, the phosphorus content in boiled eggs was found to be higher than that reported in the data from Esperance (2018), which may be attributed to the type of chicken and their feed.

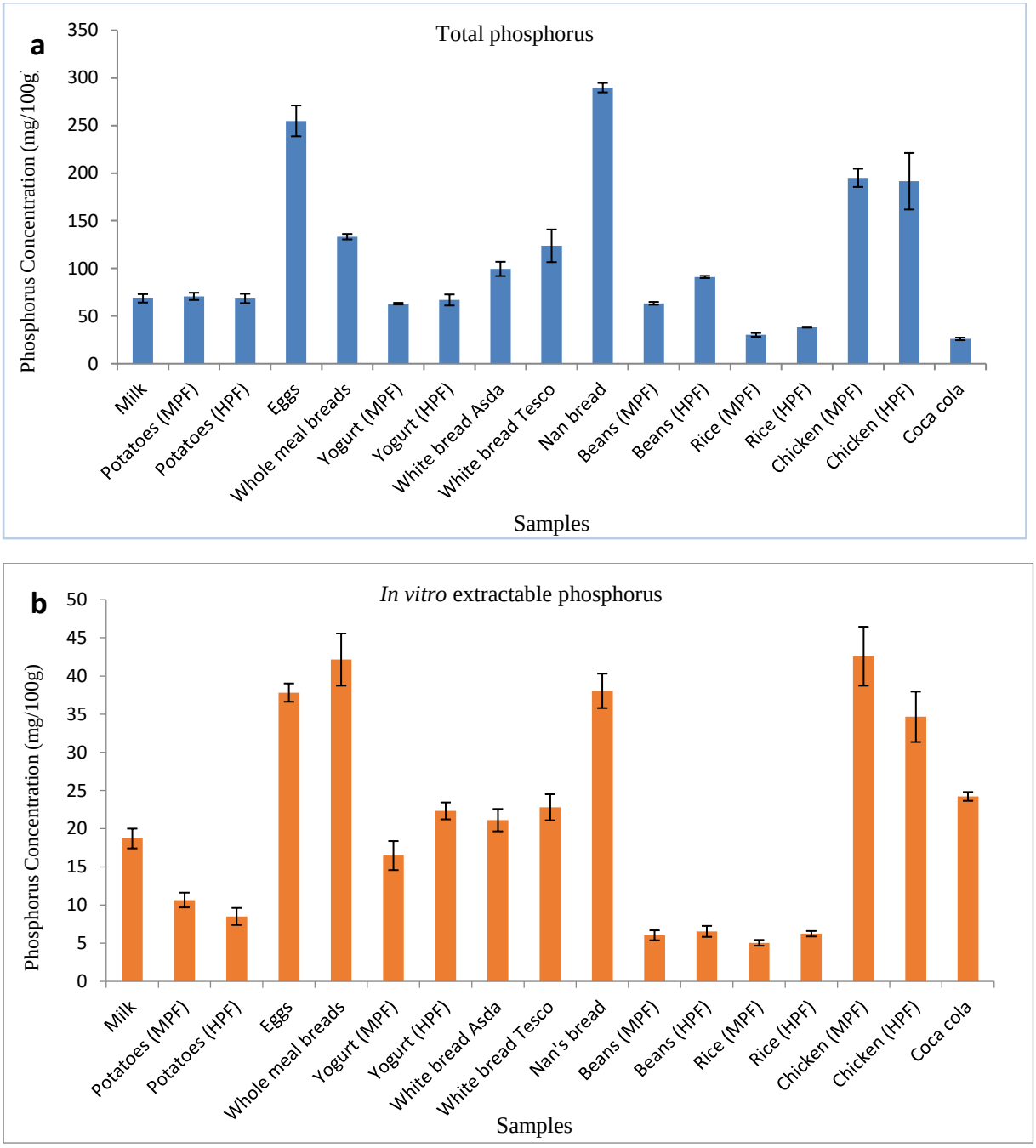

Figure 1. a: Shows total phosphorus concentration in different food samples (MPF and HPF) by using the vanadomolybdate methods; **b:** Shows the extractable amount of phosphorus content of different food samples.

A notable observation was that the phosphorus content was significantly higher in naan bread, which was prepared in a local bakery using a specific recipe unique to that establishment. These findings suggest that the phosphorus content of Nan bread may help increase phosphorus intake more effectively than other sources. Additionally, it was also observed that there was no significant difference in total phosphorus content between the white tortilla bread from Asda and the wheat and white bread from Tesco, as both types of bread contain similar ingredients. Interestingly, in this study, the manufacturing process did not affect the phosphorus content of the beans and rice samples. However, the phosphorus content in high-processed foods (HPF) was found significantly higher than that in minimally processed foods (MPF). This discrepancy may be attributed to the factors as the types of seeds, harvest season, tomato sauce, and the quantity of beans used during production.

Table 1. Shows the total phosphorus and phosphate concentration in 11 food samples with vanadomolybdate methods (measured as mg P/100 g fresh sample). Where, MPF: manufacture processed foods, HPF: home processed foods.

No	Food Samples	Process type	Total phosphorus (mg P/100g fresh sample)	Total phosphate (mg PO_4/100g fresh sample)	Ash content (g)
1	Whole milk	MPF	69 ± 0.3^{d}	211 ± 0.5^{f}	0.018 ± 0.1
	Fried Potatoes	MPF	71 ± 0.9^{d}	217 ± 0.1^{f}	0.031 ± 0.3
2		HPF	69 ± 0.12^{d}	211 ± 1^{f}	0.042 ± 0.6
3	Whole chicken eggs, boiled	HPF	255 ± 0.77^{a}	780 ± 1.2^{a}	0.015 ± 0.9
4	Whole meal breads	MPF	133 ± 1^{c}	407 ± 1.6^{c}	0.021 ± 0.6
	Yogurt	MPF	63 ± 0.83^{d}	193 ± 0.8^{g}	0.005 ± 0.2
5		HPF	67 ± 0.9^{d}	205 ± 0.7^{f}	0.009 ± 0.7
6	Wheat & white breads wraps	MPF Asda	99 ± 0.7^{c}	303 ± 0.6^{e}	0.016 ± 0.1
		MPF Tesco	124 ± 1^{c}	379 ± 0.4^{d}	0.002 ± 0.0
7	Nan bread	HPF Local	290 ± 1.9^{a}	887 ± 1.9^{a}	0.028 ± 0.1
8	beans baked in tomato sauce	MPF	63 ± 1.4^{d}	193 ± 1^{g}	0.011 ± 0.3
		HPF	91 ± 1.1^{c}	278 ± 1.2^{e}	0.015 ± 0.1
9	Basmati rice	MPF	30 ± 1.9^{d}	92 ± 0.8^{g}	0.009 ± 0.4
		HPF	38 ± 0.5^{a}	116 ± 0.8^{g}	0.012 ± 0.0
10	Chicken	MPF	195 ± 1.5^{b}	597 ± 0.9^{b}	0.014 ± 0.2
		HPF	192 ± 2.6^{b}	588 ± 1.8^{b}	0.011 ± 06
11	Soft drink (Coca cola)	MPF	26 ± 0.09^{e}	80 ± 0.9^{h}	0.007 ± 0.1

[a-h]Data are given as mean ± *SD* (n=3) and various letters are significantly different at $p \leq 0.05$.

3.2. In vitro Digestion Procedure

The purpose of this experiment was to estimate to what extent phosphorus from those food samples was available for human consumption and to show different relative availability levels between samples. Several MPF and HPF samples were analyzed through in vitro digestion procedures to calculate the relative available phosphorus content, and the obtained results of the relative available phosphorus content for all food samples are shown in (Table 2, Figure 1b). It can be observed that there were different variances between samples. In this study, the results for relative available phosphorus varied, ranging from 10% to 93% for minimally processed foods (MPF) and 7% to 37% for highly processed foods (HPF). The mean concentrations of phosphorus were also variable, ranging from 5 to 43 mg P/100 g for MPF and 6 to 38 mg P/100 g for HPF. The highest concentration of phosphorus (43 mg/100 g) was observed in chicken (MPF), while the lowest concentration (5 mg/100 g) was found in basmati rice (MPF). Figure 1b illustrates that highly processed foods (HPF) exhibited the highest relative available phosphorus content in yogurt, beans, and rice, while minimally processed foods (MPF) showed elevated levels in chicken and potatoes. The results showed that the amount of relative available P was significantly different in yoghurt and rice at (p<0.05). Table 2 indicates that the effects of home-processed foods significantly influenced the extractable phosphorus content in yogurt and rice. Three HPF (yoghurt, beans and rice) contained slightly higher relative available P than MPF. However, the relative available P content of coca cola was rather high, which may be due to food additive content. Usually, this type of beverage

includes little to no protein or other organic compounds, so the phosphorus is completely from a food additive. Therefore, Sherman (2007) states that a wide range of food additives contain phosphorus and phosphorus-containing additives are currently being added to a wide variety of processed foods such as soft drinks (Sherman, 2007). The results from Coca-Cola suggest a potential impact of food additives on patients with chronic kidney disease. In addition, the difference in relative available phosphorus content can be can be attributed to the diversity of food samples and the different processing methods employed. Moreover, the results show that the amount of relative available P was only significantly different in yoghurt and rice samples at (p<0.05).

Table 2. Shows concentration of relatively available phosphorus and phosphate in 11 food groups *in vitro* digestion methods (measured as mg P/100 g fresh sample). Where MPF: manufacture processed foods, HPF: home processed foods.

No	Food Samples	Process type	Extractable phosphorus (mg P/100g fresh sample)	Extractable phosphate (mg PO_4/100g fresh sample)	*In vitro* extractable phosphorus (%)
1	Whole milk	MPF	18.7 ± 1.2^{d}	58 ± 0.12^{d}	27
		MPF	10.6 ± 0.9^{e}	34 ± 0.33^{e}	14
2	Fried Potatoes	HPF	8.5 ± 1.1^{f}	28 ± 0.4^{f}	11
3	Whole chicken eggs, boiled	HPF	37.8 ± 1.1^{b}	116 ± 0.7^{b}	15
4	Whole meal bread	MPF	42.1 ± 1.4^{a}	129 ± 0.01^{a}	12
		MPF	16.4 ± 1.9^{d}	49 ± 1.1^{d}	23
5	Yoghurt	HPF	23 ± 1.10^{c}	70 ± 0.77^{c}	37
6	Wheat & white bread wraps	MPF Asda	22.3 ± 1.4^{c}	64 ± 2.1^{c}	16
		MPF Tesco	22.8 ± 1.7^{c}	70 ± 1.9^{c}	22
7	Nan bread	HPF Local	38 ± 2.2^{b}	116 ± 0.11^{b}	18
8	beans baked in tomato sauce	MPF	6 ± 0.6^{g}	18 ± 1.3^{g}	10
		HPF	6.5 ± 0.7^{g}	21 ± 0.66^{g}	7
9	Basmati rice	MPF	5 ± 0.3^{g}	15 ± 0.99^{g}	17
		HPF	6.2 ± 0.3^{g}	18 ± 1.4^{g}	17
10	Chicken	MPF	42.5 ± 1.8^{a}	132 ± 0.1^{a}	20
		HPF	34.6 ± 1.2^{b}	107 ± 0.12^{b}	20
11	Soft drink (Coca cola)	MPF	24.2 ± 0.5^{c}	73 ± 1^{c}	93

Interestingly, the relative available phosphorus percentage in white tortillas from Asda was higher than that in wheat and white bread from Tesco, although both types contained lower levels compared to naan bread, with values of 23%, 21%, and 38%, respectively. It was observed that the lowest amount of relative available P was found in home-processed beans 7%. This is because plant-based phosphorus (beans and legumes) is poorly digested and absorbed by humans' gastrointestinal tract, ranging from 40% to 50%, the reason is that phosphorus from plants is in the form of inorganic phytic acid (Fukagawa *et al*., 2011; Noori *et al*., 2010). In contrast, Weremko *et al*. (1997) reported that the availability of phosphorus in plants varies from 10% to 60%, which can be attributed to the presence of phytate.

In this study, the available P content (percentage) was generally lower than those reported in the literature with the exception of Coca-Cola. It is evident that the in vitro methods include gastrointestinal digestion using pepsin at pH 2 for 2 hours, followed by digestion with pancreatin-bile extract during the intestinal stage at pH 7 for an additional 2 hours. The results

indicate that incubation time and pH may have little effect on the digestion process. The variation in pH conditions and the unstable incubation times within the context of this study are the primary factors resulting in the lower available phosphorus content (percentage). However, it was found that the digestibility increased with increasing incubation time (Miller *et al*., 1981). On the other hand, mineral absorption occurs in the earlier part of the small intestine, therefore the reproduction of the situation existing in the small intestine is the most important step for in vitro digestion procedure (Shiowatana *et al*., 2006). It should be noted that the aforementioned situation may not be appropriately applicable to in vitro methods; therefore, pH and incubation time may affect digestive enzymes as well. This may be attributed to the digestive enzymes not being sufficiently active to properly digest the samples; however, the effects could differ if the samples were consumed by humans.

3.3. Phosphorus with Their Impact on Advanced-Stage CKD Patients

The comparison between the total phosphorus and in vitro extractable phosphorus showed that the amount of total and relative available P was significantly different in all food samples at (p<0.05). The strong association between diet and health underscores the critical role of dietary guidance in managing and potentially preventing phosphorus imbalances in advanced-stage chronic kidney disease (CKD). It is a leading cause of morbidity and mortality throughout the world. Returning to the two primary findings, it can be concluded that these food samples are suitable for consumption by patients with end-stage chronic kidney disease (CKD), specifically those undergoing dialysis. Patients with advanced-stage chronic disease require specific dietary restrictions to limit the build-up of extra phosphorus in the body. Winger *et al*., (2012) show that control of serum phosphate is achieved by reducing dietary P intake. Moreover, Uribarri (2001) indicated that dialysis alone is insufficient to prevent elevated serum phosphorus levels in patients without dietary restriction. Generally, patients with advanced-stage CKD can face varying lifestyle challenges such as dietary modification as well. Recommendations for these patients are often complex and depend on the stage of the disease and the presence of coexisting medical conditions, such as diabetes (Winger *et al*., 2012). Dietary restrictions are recommended for dialysis patients, particularly regarding protein intake. However, this may lead to malnutrition over time, as high-protein foods are a major source of dietary phosphorus. (Shinaberger *et al*., 2008). Consequently, dietary phosphorus restriction must be balanced with adequate protein intake to prevent additional health complications for the patient. This study demonstrates that home-processed foods (HPF) generally have a higher total phosphorus content but exhibit lower or comparable available phosphorus percentages compared to manufactured processed foods (MPF). For example, home-processed potatoes (HPF) contain 69 mg P/100 g, with only an estimated 11% available, while manufactured processed potatoes (MPF) contain 71 mg P/100 g, with 14% available (Table 3 and 4). It is also obvious that relative available P significantly affects serum PO4 levels in patients with end-stage CKD. Additionally, manufactured processed foods (MPF) such as chicken and soft drinks may contain phosphorus additives that are significantly higher than those found in home-processed foods (HPF). Inorganic phosphorus present in additive foods is absorbed at a rate exceeding 90% (González-Parra *et al*., 2012; Rosenberg, 2000). This study demonstrated that 93% of the phosphorus content in coca cola was available. For this reason, patients with stage 3-5 CKD are advised to avoid using MPF such as coca cola, because clinically it is a leading cause of significant improvement in serum phosphate.

3.4. Measuring Dietary Intake in The Context of Food Analysis

Dietary intake has an important role in increasing and decreasing phosphate content in the blood. The analyzed food samples were categorized into manufactured processed foods (MPF) and home processed foods (HPF) for the purpose of this study. In addition, typical daily diet for an individual was calculated based on both manufactured processed foods (MPF) and home-processed foods (HPF) over two different days. Hence, the recommended daily phosphorus intake for a patient with advanced-stage CKD ranges between (550 – 1100 mg/day) (Kooienga,

2007). The average diet in Europe contains approximately 1,000 to 1,500 mg of phosphorus per day (EFSA, 2019). Table 3 shows each serving of (MPF) samples contains (1237 mg) of phosphorus per day, which is slightly higher than the clinically accepted range of 550 to1100 mg/day for patients with end-stage CKD while indicating lower relative available P content (329 mg). In contrast, Table 4 demonstrates that each serving of home-processed food (HPF) samples contains 1,717 mg of phosphorus per day, which exceeds the clinically accepted range of 550 to 1,100 mg/day for patients with end-stage chronic kidney disease (CKD). However, it indicated higher available P content than the MPF. The difference between manufactured processed foods and home-processed foods may be due to differences in ingredients. The home process ingredients were found to be more effective in increasing daily P intakes compared to MPF, particularly in Nan bread, which contained the highest amount of phosphorus among all other samples. Based on the results obtained in this study, it is proposed that dietary restrictions for both manufactured processed foods (MPF) and home-processed foods (HPF) are influential and essential for balancing phosphate levels in the body. There are considerably fewer data and research published on the total P and relative available P content of (MPF) and (HPF).

On average patients with end-stage renal disease are facing many challenges in their daily life with dietary restrictions, and the lack of information about dietary content. For example, naan bread is a popular type of bread in many countries worldwide, typically prepared in local bakeries without any labeling or nutritional information. Many people consider Nan bread to be a natural source of nutrients. This leads to the conclusion that it is not accurate to assert that all home-processed foods have high phosphorus content. In addition, eggs are one of those food sources that is prepared at home when was contains a high amount of phosphorus. These results suggest that is important to explore alternative approaches to prevent complications in patients with end-stage chronic kidney disease (CKD). Instead of solely advising patients to avoid manufactured processed foods, there should be a focus on producing specialized foods through manufacturers, while encouraging greater reliance on home-processed foods.

Table 3. shows mean and percentage of the total and relative available phosphorus in manufacture processed foods (MPF) for one-day meals of a normal individual. Where 1 cup milk: 250g, 1oz potatoes: 28.42g, 1 slice whole bread: 22g, 1 slice wheat bread: 23g, 1cup beans: 253g, 1cup rice: 164g, 1oz yoghurt: 227g, chicken breast:140g, Coca-Cola: 370g.

Food samples	Total P (mg/100g)			*In vitro* extractable P (mg/100g)		*In vitro* extractable P %
	mg P/100g	Rate	Daily/mg	mg P/100g	Daily/mg	%
Whole milk	69	1 cup	173	19	48	27
Potatoes	71	2 oz	40	11	6	14
Whole meal bread	133	4 Slice	122	42	39	12
Yoghurt	63	1/8-oz container	143	16	36	23
Wheat &white bread (Asda)	99	2 Slice	40	21	9	16
Beans in tomato sauce	63	1 cup	159	6	15	18
Basmati rice	30	4 cup	197	5	33	17
Chicken breast	195	140 g	273	43	60	20
Coca cola	26	370 mL	90	24	83	93
Total intake			1237		329	27%
Daily intake for a normal individual in one day			1237 (mg/day)			27%

Table 4. presents the mean and percentage of total and relative available phosphorus in home-processed foods (HPF) for a typical day's meals for an individual. Where 1 cup meal: 250g, 1oz potatoes: 28.42g, 1 Nan: 100g, 1cup beans: 253g, 1cup rice: 164g, 1oz yoghurt: 227g, 1 large egg; 50g, chicken breast: 140g, coca-cola: 370g.

Food samples	Total phosphorus (mg/100g)			*In vitro* extractable P (mg/100g)		*In vitro* extractable P %
	mg P/100 g	Rate	Daily	mg P/100 g	Daily/mg	%
Whole milk	69	1 cup	173	19	48	27
Potatoes	69	2 oz	39	9	5	11
Yoghurt	67	8-oz container	152	23	52	37
Nan bread	290	1 Nan	290	38	38	18
Beans in tomato sauce	91	1 cup	230	7	18	7
Basmati rice	38	4 cup	249	6	39	17
Chicken breast	192	140g	269	35	49	20
Coca-cola	26	370 mL	90	24	83	93
Eggs	255	2 large	225	38	38	15
Total intake			1717		370	27
Daily intake for a normal individual in one day		1717 (mg/day)			27%	

4. CONCLUSION

This study aimed to analyze the phosphorus content in two types of processed foods to evaluate their effects on dialysis patients. Most food samples in this study contained a reliable amount of total phosphorus with the exception of Nan bread and eggs. The highest concentration of total phosphorus was found in naan bread, whereas the lowest concentration was found in Coca-Cola. The availability of phosphorus differed considerably depending on the type of food. The lowest values were observed in basmati rice (MPF), while the highest values were found in chicken breast (MPF); both were below the expected range of 50-60%. The high phosphorus intake could be the main reason for the increased PO4 level in patients with end-stage chronic diseases. Therefore, the findings of this study provide valuable information for individuals with advanced-stage chronic kidney disease (CKD), helping them make informed dietary choices to protect their health and avoid foods that may exacerbate their condition. Further in vivo research is needed to evaluate the effects of home-processed foods and manufactured processed foods on patients with end-stage chronic kidney disease with a particular focus on foods such as Nan bread and egg that include high total P content. Patients with end-stage chronic kidney disease are recommended a varied diet consisting of both animal and plant products high in phytate with enough protein content as well. The consumption of processed foods should be restricted due to their high content of food additives. Additionally, certain home-prepared foods, such as naan bread and eggs, should also be consumed with caution. Patients are also recommended to increase their knowledge and awareness of the phosphorus content in commonly consumed foods. their knowledge about dietary phosphorus content in common foods.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

All authors contributed equally to the writing of the article. In addition, all authors have read and agreed to the published version of the article.

Data Availability

Data supporting the findings of this study are available from the corresponding author upon request.

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Research Article

A comparative analysis of the effects of different drying and storage techniques on the phenolic content of *Pistacia atlantica* leaves

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Abstract: This study presents a comparative analysis of the effects of different drying and storage techniques on the phenolic content of *Pistacia atlantica* leaves in both whole and ground states. Four drying methods were evaluated: direct sunlight, a ventilated drying chamber, shade, and obscurity. Additionally, three storage conditions were assessed: direct sunlight, shade, and freezing. The concentrations of total phenolics, total flavonoids, and tannins were quantified, and antioxidant activity was evaluated using the DPPH radical scavenging assay. Our findings indicate that drying in obscurity and shade yielded the highest levels of total phenolics (369.45±2.12 and 362.78±1.36 mg GAE/g DM), total flavonoids (54.34±0.95 and 55.68±1.25 mg QE/g DM), and tannins (36.35±0.91 and 33.80±0.79 mg CE/g DM), correlated with strong antioxidant activity (1.85±0.01 and 1.92±0.01 µg/mL, respectively), particularly when the leaves were stored whole. The results emphasize that controlled drying methods (ventilated chambers and darkness), along with storing the leaves whole and under freezing conditions, are optimal for preserving the antioxidant activity and phenolic content of *P. atlantica* leaves. Freezing proved to be the most effective storage condition for preserving phenolic compound concentrations and their associated antioxidant properties. Overall, the study highlights that the strategic selection of drying techniques and storage conditions is critical for optimizing the preservation of phenolic compounds and antioxidant activity in plant materials.

1. INTRODUCTION

Medicinal plants have been utilized across various fields for centuries, including traditional medicine, pharmaceuticals, cosmetics, nutrition, agriculture, and environmental restoration. They yield active compounds for pharmaceutical applications, natural ingredients for skincare, essential nutrients for well-being, biopesticides for crop protection, and contribute to ecological balance. Medicinal plants are critical subjects of scientific inquiry, leading to the discovery of new therapeutic compounds and expanding our understanding of their mechanisms of action. Medicinal plants are often used in their dried form rather than fresh. Drying the plants helps to enhance their shelf life, improve their storage stability, and concentrate their active compounds. The quality and effectiveness of dried medicinal plants heavily rely on the precise drying and

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storage conditions employed. It is crucial to employ suitable drying techniques, along with implementing proper storage conditions such as controlled temperature, humidity, and protection from light. By ensuring these optimal conditions, the integrity of the plant's constituents can be preserved, guaranteeing the long-term potency and efficacy of the dried medicinal plants.

Several studies have been conducted on the effects of drying techniques like air-drying, freeze-drying, and shade-drying, as well as on storage conditions such as freezing, canning, and other methods (Bettaieb Rebey *et al*., 2020; Czarniecka-Skubina, 2002; Kumar *et al*., 2022; Lin *et al*., 2020; Roshanak *et al*., 2016; Vega-Gálvez *et al*., 2011).

The main objective of this study was to assess the impact of various drying techniques, including direct sunlight, ventilated drying chamber, shade, and obscurity, as well as different storage methods such as direct sunlight, shade, and freezing, on the phenolic content and antioxidant activity. The chosen example was the leaves of *P. atlatica*, which is a species that has been extensively studied (Amel *et al*., 2016; Belyagoubi-Benhammou *et al*., 2015; Belyagoubi *et al*., 2016; Benhammou *et al*., 2008; Benmahieddine *et al*., 2021, 2023; Toul *et al*., 2017, 2022), focusing on various aspects. However, the specific effects of different drying and storage techniques on the phenolic content of these leaves have not been previously investigated. This study aims to fill this research gap and provide insights into the impact of drying and storage conditions on the phenolic composition of *P. atlantica* leaves.

2. MATERIAL and METHODS

2.1. Plant Material

The leaves of *P.atlantica* were randomly harvested, without regard to exposure, from the municipality of Igli, wilaya of Beni Abbass, located in the southwest of Algeria, in March 2021. Botanical identification was performed in the Laboratory of Valorization of Plant Resources and Food Security in Semi-Arid Areas, Department of Biology, Faculty of Nature and Life Sciences, Tahri Mohammed University of Bechar.

2.2. Drying Conditions

As shown in Figure 1, the harvested leaves were divided into four distinct groups, with each group subjected to a different drying method. The leaves were deemed dry when they became brittle or partially brittle, depending on the drying technique, and their color transitioned from green to yellow-green.

The first group of leaves was dried under direct sunlight (S), exposing them to natural environmental conditions, including variable temperatures and humidity levels, for approximately 3 days (they were moved to a well-ventilated indoor area during the night to prevent moisture reabsorption. The drying process resumed the following day under direct sunlight until the desired dryness was achieved). The second group was dried inside a ventilated chamber (laboratory oven) set at a constant temperature of 30°C (Dc), ensuring a controlled drying environment with minimal exposure to external factors, over 2 days. The third group was shade-dried at room temperature (Sh), allowing for a slower drying process under indirect light, which took about 5 days to complete. The fourth group was dried in a completely dark place (obscurity) at room temperature (O), minimizing light exposure to prevent degradation of light-sensitive compounds, for 7 days. Once the drying process was complete, a portion of each group was analyzed to assess their phenolic content and antioxidant activity.

2.3. Storage Conditions

The four groups of dried leaves were divided into three subgroups, and each subgroup from each group was stored for 30 days under different conditions: in the freezer at -18°C (F), under shade at room temperature and relative humidity (Sh), or exposed to direct sunlight (S). Additionally, each leaf subgroup was further stored in two different forms: entire leaves (E) and ground leaves (G), as depicted in Figure 1.

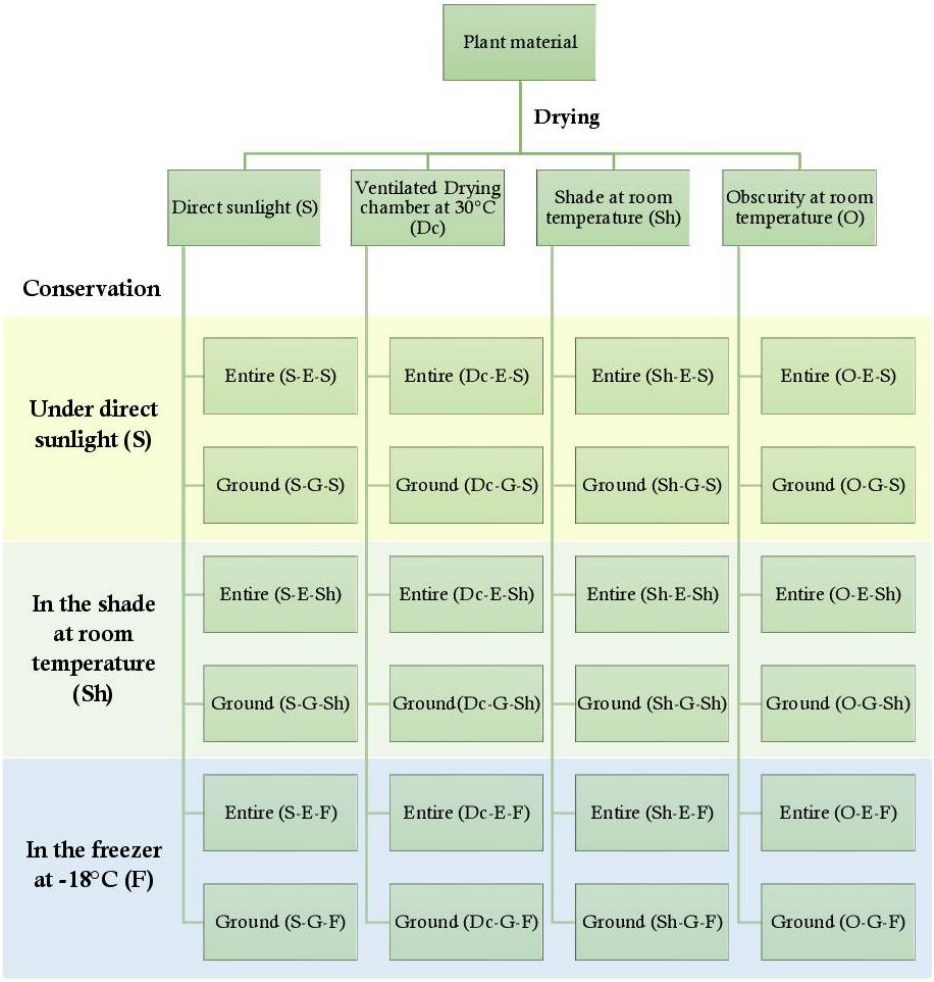

Figure 1. Drying and storage protocol.

2.4. Preparation of Extracts

Entire leaves were ground using a basic cutter mill (IKA A10, IKA-Werke, Germany) into a fine powder just before extraction, without considering milling time and speed. Only the necessary quantity was processed to avoid browning and potential degradation of active compounds. From each subgroup, 1 g of powdered leaves was macerated in 20 mL of methanol for 24 hours at room temperature. The methanolic solution was filtered and then evaporated to dryness at 60°C using a standard rotary evaporator. The dry residue was resolved in some milliliters of methanol and stored in the refrigerator for later use (Toul *et al*., 2017).

2.5. Total Phenolic Content (TPC)

A volume of 200 µl of each crude extract was added to 1 mL of 10 times diluted Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate (Na_2CO_3) at 7.5%. The mixture was incubated at room temperature for 30 minutes, and the absorbance was recorded at 765 nm of wavelength against a blank (Singleton & Rossi, 1965). The results were expressed in milligram equivalents of gallic acid per gram of dry matter.

2.6. Total Flavonoid Content (TFC)

A volume of 500 µL of different concentrations of extract was mixed with 1500 µL of distilled water. At time zero, 150 µL of 5% $NaNO_2$ was added to the mixture. After 5 min, 150 µL of 10% $AlCl_3$ were introduced. After being incubated for 6 min at room temperature, 500 µL of NaOH (1M) were added. Immediately, the mixture was thoroughly shaken, and the absorbance was recorded at 510 nm against the blank. The total flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry matter (Zhishen *et al*., 1999).

2.7. Tannin Content (TC)

An aliquot of 50 µl extract was added to 1500 µl of a 4% (m/v) vanillin/methanol solution. After being stirred, 750 µl of concentrated hydrochloric acid (HCl) was added. The absorbance was measured at 550 nm after 20 minutes of incubation (Julkunen-Tiitto, 1985). Tannin content was expressed as milligram equivalents of catechin per gram of dry matter.

2.8. DPPH Radical Scavenging Assay

The antioxidant activity of the different extracts was assessed through their scavenging potential against the DPPH radical. Fifty microliters of extract were mixed with 1950 µL of 0.025 g/L DPPH methanolic solution. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The reaction of the DPPH radical was estimated by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the following equation:

$$RSA(\%) = \frac{A_C - A_S}{A_C} \times 100$$

Where A_C is the control's absorbance and A_S is the absorbance of the tested extract. Extract concentration providing 50% inhibition (IC50) was calculated from the graph plotting inhibition percentages against extract concentrations. BHA and quercetine were used as reference compounds.

2.9. Statistical Analysis

Experimental data were statistically analyzed using one-way analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), and Pearson correlation to evaluate the impact of drying methods, conservation and storage conditions on the antioxidant activity and the concentrations of bioactive compounds in *P. atlantica* leaf extracts. One-way ANOVA was employed to assess the significance of differences in IC50 values for each individual parameter. MANOVA was utilized to evaluate the combined effects of drying techniques, conservation and storage conditions on IC50, total phenolic content (TPC), total flavonoid content (TFC), and tannin content (TC). Pearson correlation analysis was conducted to explore the relationships between IC50 values and levels of TPC, TFC, and TC. Data are presented as means ± standard deviation (*SD*). All statistical analyses were performed using SPSS version 25.0, with significance determined at $p \leq 0.05$, and graphical representations were generated using Microsoft Excel 2021.

3. FINDINGS and DISCUSSION

The results of this study are presented using a pseudo-Pareto chart, which clearly illustrates the relative significance of each drying technique, drying state, and storage condition. This visual representation, organized in descending order of impact, allows for the identification of the most influential factors affecting the phenolic composition and antioxidant activity of *P. atlantica* leaf extracts. This approach provides a comprehensive overview, enhancing the understanding of how various external factors influence the bioactive properties of the plant material.

3.1. Total Phenolic, Flavonoid, and Tannin Content

As shown in Figure 2, total phenolic content across the main drying techniques decreased in the following order: O>Sh>Dc>S. Specifically, leaves dried under direct sunlight exhibited a loss in total phenolics ranging between 22% and 37% compared to the other techniques. Figure 2 also reveals that the use of direct sunlight (S) as a drying or preservation technique consistently reduced total phenolic content. In contrast, leaves stored whole and frozen maintained the highest phenolic content, followed by those stored whole in the shade.

These findings are consistent with those reported by Bettaieb Rebey *et al*. (2020), where shade drying yielded a total phenolic content 1.4 times higher than that observed with ventilated

drying chamber. Additionally, other studies have indicated that drying leaves in Dc leads to significantly higher total phenolic content compared to sun drying and shade drying, where the total phenolic content being 1.6 times higher for leaves dried in Dc and 1.9 times higher compared to shade-dried leaves (Roshanak *et al*., 2016). The losses in total phenolic content (TPC) observed during the drying processe may result from several factors, including binding of polyphenols with other compounds or alterations in their chemical structure, which may reduce their extractability and quantification by available methods. However, it is important to note that the impact of drying on TPC can vary, and in some cases, drying may even lead to an increase in TPC (Vega-Gálvez *et al*., 2011).

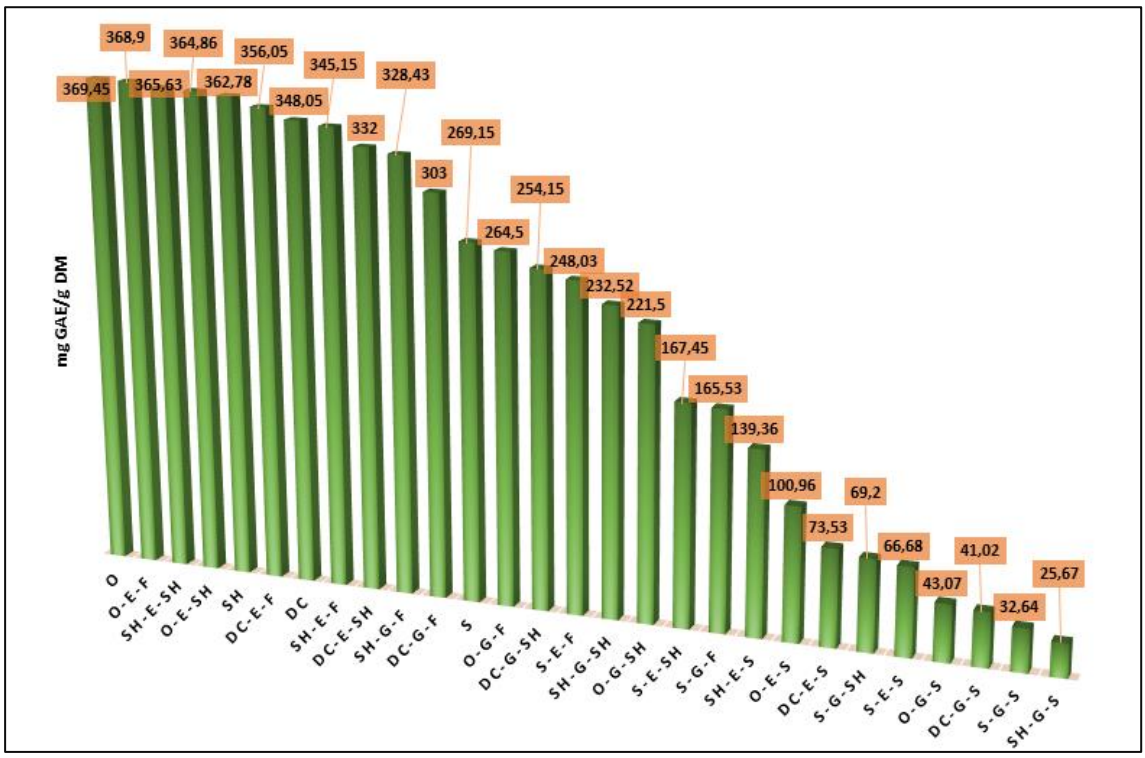

Figure 2. Decreasing total phenolic content of *P. atlantica* leaf extracts.

Figure 3 illustrates the variation in total flavonoid content (TFC) and tannin content (TC) in *P. atlantica* leaf extracts subjected to different drying techniques, storage conditions, and preservation methods. Flavonoids and tannins exhibited different responses to the drying techniques employed. Total flavonoid content decreased in the following order: Sh > O > Dc > S. Leaves dried under direct sunlight (S) showed a significant decrease in flavonoid levels, with losses ranging from 46% to 56%. These results are consistent with the findings of Roshanak *et al*. (2016), who reported that sun and oven drying caused respective losses of 20% and 25%, compared to shade drying. In contrast, tannins exhibited a different response, following the order: O > Sh > S > Dc. Interestingly, leaves dried under direct sunlight displayed a smaller decrease in tannin levels ranging from 9% to 25%. This can be attributed to the heat stability of tannins, as suggested by Chung *et al*. (1998), and further confirmed by Kumar *et al*. (2022). Previous studies have shown that ventilated drying chambers may lead to losses in both flavonoids and tannins due to increased heat exchange and air circulation, which can enhance oxidation and degradation processes (Hamrouni-Sellami *et al*., 2013; Hu *et al*., 2021; Mediani *et al*., 2014).

Additionally, while the effects of drying techniques on phenolic compounds are well-documented, there is limited information regarding the impact of preservation methods, such as freezing, on phenolic content in vegetables and fruits. Freezing is widely recognized as an effective preservation method, capable of maintaining the active constituents of raw plant materials over extended periods. This approach has demonstrated efficacy in preserving bioactive compounds, with potential applications in pharmaceuticals, cosmeceuticals, and

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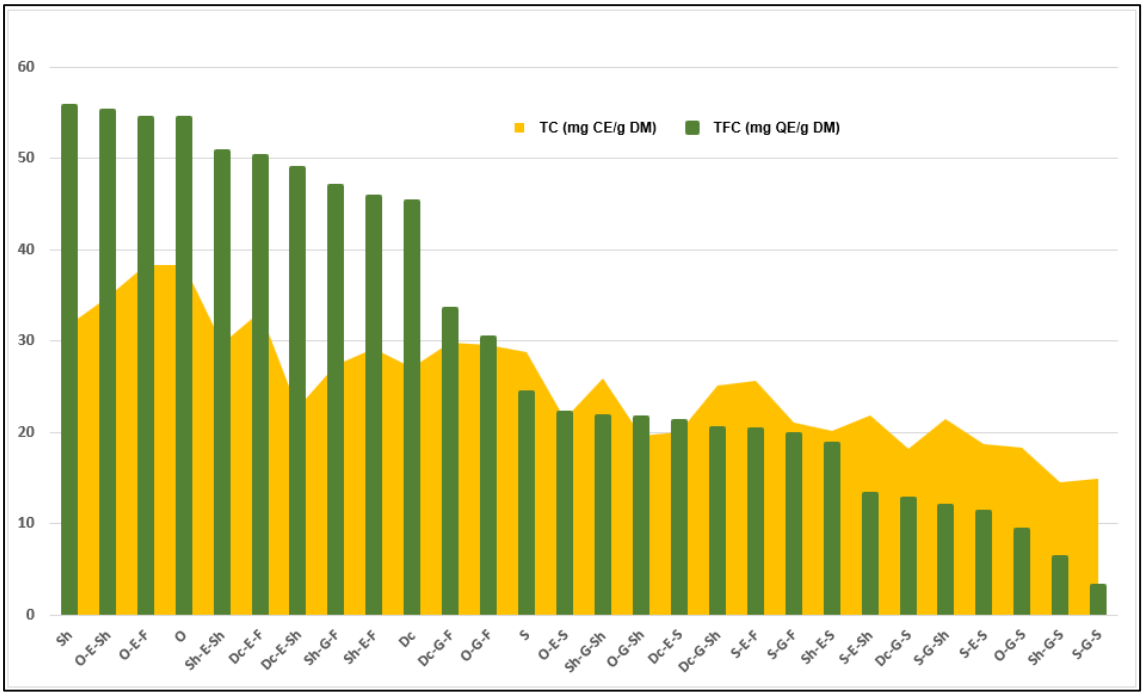

Figure 3. Combined total flavonoid and tannin content of *P. atlantica* leaf extracts.

3.2. Antioxidant Activity

The results of the assessment of antioxidant activity, measured through DPPH scavenging potential, are presented in descending order of IC50 values (µg/mL) and supplemented by graphs illustrating the levels of total polyphenols, flavonoids, and tannins. This integrated representation effectively highlights the most influential factors, enabling a clear determination of their relative significance. A lower IC50 value in the DPPH assay indicates stronger antioxidant activity, reflecting the compound's ability to scavenge free radicals at lower concentrations (Amel *et al*., 2016; Toul *et al*., 2022).

Figure 4 clearly shows a dose-dependent relationship between antioxidant activity and total phenolic content. The results emphasize the impact of different drying methods on this relationship. Specifically, the leaves dried in obscurity or under shade exhibited the lowest IC50 values, 1.85±0.03 and 1.92±0.05 µg/mL, respectively, indicating the highest antioxidant activity, even when compared to the reference compounds, BHA and quercetine (2.09±0.02 and 5.21±0.15 µg/mL, respectively). These leaves also exhibited the highest phenolic contents. This correlation highlights the influence of drying methods on both the antioxidant activity and phenolic content of the extracts, as evidenced by the graph progression with the exposure of other factors: ventilation, grinding, and direct sunlight, where the IC50 values increased from 13.64±1.66 to 225.64±7.48 µg/mL.

Regarding the storage conditions, samples stored in the freezer exhibited slightly higher antioxidant activity than those stored under shade, and significantly higher activity those stored under direct sunlight. Lin *et al*. (2020) reported a 97.41% decrease in DPPH scavenging ability when stored at 37°C for 3 days, a 54.36% decrease at 25°C, and only a 5.15% decrease when stored at 4°C.

The statistical analysis revealed significant effects of drying methods, conservation and storage conditions on the antioxidant activity and bioactive compound content of *P. atlantica* leaf extracts. One-way ANOVA showed significant differences in IC50 values across various

conditions ($F = 16.71$, $p < 4.86\times10^{-9}$). Drying in a ventilated chamber (Dc) and in obscurity (O) preserved the highest levels of TPC, TFC, and TC, resulting in the lowest IC50 values, which indicate high antioxidant activity. In contrast, drying under direct sunlight (S) caused significant losses in bioactive compounds, resulting in higher IC50 values.

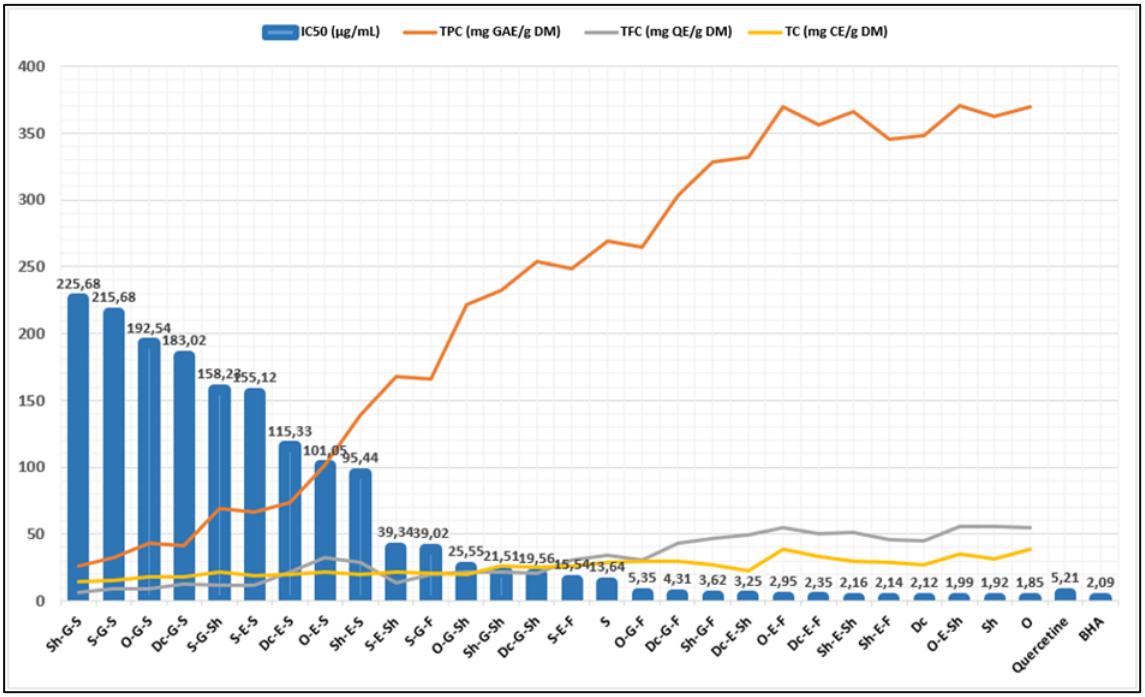

Figure 4. Decreasing IC50 Correlated with TPC, TFC, and TC in *P. atlantica* leaf extracts.

Multivariate analysis of variance (MANOVA) showed significant multivariate effects for drying techniques (Wilks' $\lambda = 0.0012$, $F(48, 83.29) = 3.04$, $p < 0.05$), conservation (Wilks' $\lambda = 0.0021$, $F(32, 66.54) = 2.65$, $p < 0.05$), and storage conditions (Wilks' $\lambda = 0.0033$, $F(16, 54.12) = 2.17$, $p < 0.05$), confirming the combined influence of these factors on IC50, TPC, TFC, and TC. Pearson correlation analysis further revealed strong inverse relationships between IC50 and TPC ($r = -0.865$, $p < 0.05$), IC50 and TFC ($r = -0.864$, $p < 0.05$), and IC50 and TC ($r = -0.869$, $p < 0.05$), indicating that higher levels of these compounds are associated with higher antioxidant activity.

These findings are consistent with previous studies on the preservation of bioactive compounds. Vega-Vega-Gálvez *et al*. (2009) reported that air-drying temperature significantly influences the physicochemical properties and antioxidant capacity of red pepper, with controlled drying conditions better preserving phenolic and flavonoid contents. This is in agreement with the current study, which indicates that drying in a ventilated chamber or in darkness is more effective. Similarly, Ratti (2001) revealed that freeze-drying is highly effective for preserving the quality of high-value foods by minimizing enzymatic and oxidative degradation. This supports the finding in the present study that freezing is the most effective storage method for preserving bioactive compounds.

Chan *et al*. (2009) demonstrated that different drying methods significantly impact the antioxidant properties of ginger species, with controlled environments better preserving bioactive compounds, which aligns with the current findings that drying in controlled conditions (ventilated chamber and darkness) preserves antioxidant properties.

4. CONCLUSION

The study highlights that to preserve the antioxidant activity and bioactive compound integrity in *P. atlantica* leaves, it is critical to employ specific post-harvest handling practices. The optimal preservation involves drying in a ventilated chamber or in darkness, which minimizes photodegradation and oxidative loss of phenolic compounds. Additionally, storing the leaves

intact, rather than ground, helps in retaining cellular integrity and reducing exposure to oxidative processes. Conservation under freezing conditions further prevents enzymatic activity and degradation of sensitive bioactive compounds. Together, these methods preserve the levels of phenolic, flavonoid, and tannin contents, thereby maximizing the leaves' therapeutic and antioxidative potential. Among the techniques assessed, the most effective approach is drying the plant material in a ventilated chamber under darkness, keeping the leaves whole, and storing them in the freezer after complete drying.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Fethi Toul: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Marwa Ballou**: Investigation, Methodology. **Marwa Kessou**: Investigation, Methodology.

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Research Article

Investigation of biological interactions in *Euphorbia rigida* extract using molecular docking

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Abstract: In this study, the antioxidant activity, phenolic content, and antimicrobial properties of *Euphorbia rigida* aerial parts methanol extract were investigated. The extract demonstrated significant antioxidant activity with a DPPH radical scavenging activity IC_{50} value of 919.46 µg/mL. The iron chelating activity was characterised by an IC_{50} value of 4.24 mg/mL, with total phenolic content measured at 11.96 mg GAE/g extract DW and total flavonoid content at 26.83 mg QE/g extract DW. The antimicrobial evaluation compared the *E. rigida* aerial parts methanol extract to standard drugs such as Ampicillin, Chloramphenicol, and Ketoconazole. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranged from 12.5 mg/mL to >50 mg/mL. The extract exhibited strong antibacterial effects with MIC and MBC values of 25 mg/mL for *E. coli* and 12.5 mg/mL for *B. cereus*. Additionally, while some antifungal activity was observed against *C. albicans*, it was less effective than Ketoconazole. GG-MS analysis identified Guanosine as the most abundant compound in the extract, accounting for 35.78% of the total area. Molecular docking studies with phosphatidylinositol-specific phospholipase C showed that Guanosine had the strongest binding affinity with a binding energy of -5.0 kcal/mol, forming multiple interactions. Neophytadiene and Dihydroxyacetone exhibited weaker binding affinities and fewer interactions. Toxicity assessments indicated low toxicity for the extract's components, with LD50 values of 2200 mg/kg for Dihydroxyacetone, 13 mg/kg for Guanosine, and 500 mg/kg for Neophytadiene. In summary, the study sought to elucidate the antimicrobial potential and biological interactions of *E. rigida* aerial parts methanol extract.

1. INTRODUCTION

Medicinal plants are those with organs containing substances that affect living organisms. Medicinal plants are considered one of the oldest human achievements for treating diseases. Throughout the development of human civilisations, there has consistently been a close relationship between humans and plants (Jafari-Sales *et al*., 2019). Although many plant species have been identified to date, a plethora of new and valuable plant resources remain to be discovered (Sales, 2020). Though only partially identified so far, these chemical components can serve as drugs and unique starting points to produce pharmaceutical analogues and as

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valuable tools for enhancing our understanding of biological phenomena (Jafari-sales *et al*., 2019). Due to the side effects of antibiotics and the increasing resistance of microorganisms, the use of medicinal plants for combating bacterial infections has become more prevalent (Gholami *et al*., 2019). Due to the increasing problem of antibiotic resistance, attention has shifted towards biologically active compounds obtained from plants. These compounds can potentially serve as new and effective sources of antibacterial and antifungal activities (Erfan & Marouf, 2019; Maiyo *et al*., 2010). The antimicrobial properties of plants are associated with their ability to produce various secondary metabolites with complex structures (Matasyoh *et al*., 2009).

Bacillus cereus is one of the leading etiological agents of toxin-induced foodborne diseases. Its widespread presence in various environments, ability to form spores, and capacity to adapt to different conditions and produce harmful toxins make this pathogen a significant health hazard that should not be underestimated. Food poisoning caused by *B. cereus* can manifest as emetic or diarrheal syndrome. The final harmful effects are not only dependent on the toxins and strains. However, they are also influenced by stress responses, accessory virulence factors, phenotypic characteristics under extrinsic, intrinsic, and specific food conditions, and the host environment (Jovanovic *et al*., 2021).

The genus *Euphorbia* is the largest in the Euphorbiaceae, comprising approximately 2000 species, with 91 species found in Turkey (Özbilgin *et al*., 2012). These plants contain latex and are distinguished by their unique flower structures. *Euphorbia* species include many terpenoid compounds, such as monoterpenes, sesquiterpenes, diterpenes, triterpenes, and steroids. Many of these compounds have been studied for their toxicity and potential therapeutic effects, including antimicrobial, anti-inflammatory, anticancer, and antioxidant properties; some have historically been used as medicines (Gherraf *et al*., 2010).

The primary objective of this study is to comprehensively evaluate the antimicrobial activity of *E. rigida* aerial parts methanol extract. The study aims to determine the extract's effectiveness against various bacterial and fungal pathogens. The extract's antioxidant capacity and phenolic content will also be analysed to assess their contributions to microbial efficacy. Furthermore, the chemical identification of bioactive compounds present in the extract will be performed, and the toxicological profiles of these compounds will be evaluated. In summary, the study seeks to elucidate the antimicrobial potential and biological interactions of *E. rigida* aerial parts methan**ol** extract.

2. MATERIAL and METHODS

2.1. Collection of The Plant

E. rigida specimens were collected from the Asarkale area in Bafra, Samsun, and the entire plant, including both aboveground and underground parts, was harvested in June. Dr. Alper DURMAZ identified the species using the "Flora of Turkey," the latest nomenclature and epithet were confirmed through the POWO (Plants of the World Online) database. The herbarium material was catalogued under accession number OMUB-3184 in the Herbarium of the Biology Department at Ondokuz Mayıs University.

2.2. Plant Extraction

The methanol extract of *E. rigida* aerial parts was dried in an oven at 40°C and then ground into a powder using a blender. The maceration method suggested by (Aytar, 2024) was employed for extraction. The flowers, dried at 40°C, were extracted with methanol at room temperature for two days in a dark environment. The obtained extracts were filtered with filter paper. After filtration, the solvent was evaporated under reduced pressure at 40°C using a rotary evaporator, and solid extracts were stored at 4°C.

2.3. Spectroscopic Analysis of Secondary Metabolites

2.3.1. *Total phenolic content*

Total phenols were measured using the Folin-Ciocalteu method developed by (Singleton *et al*., 1999). Plant extracts were diluted to a concentration of 1 mg/mL. 0.5 mL was taken from these solutions and mixed with 2.5 mL of Folin-Ciocalteu reagent and 2 mL of 7.5% $NaHCO_3$. The Folin-Ciocalteu reagent had been previously diluted tenfold with distilled water. The mixture was incubated at 45 °C for 15 minutes. After the incubation period, the absorbance was measured at 765 nm using a UV spectrophotometer. Total phenol content was determined as gallic acid equivalents dry weight extract (mg GAE/g extract DW), and these values are presented as the means of triplicate analyses.

2.3.2. *Total flavonoid content*

The total flavonoid content was measured following a previously reported spectrophotometric method (Dewanto *et al*., 2002). The procedure was as follows: Extracts of each plant material (1 mL containing 0.1 mg/mL) were diluted with water (4 mL) in a 10 mL volumetric flask. Initially, 5% $NaNO_2$ solution (0.3 mL) was added to each volumetric flask. At the 5 minute, 10% $AlCl_3$ (0.3 mL) was added. At the 6-minute, 1.0 M NaOH (2 mL) was added. Water (2.4 mL) was added to the reaction flask and mixed well. The absorbance of the reaction mixture was read at 510 nm using a UV spectrophotometer.

The total flavonoid content was quantified as quercetin equivalents (QE) per gram of dry-weight extract (mg QE/g extract DW). All measurements were performed in triplicate, and the results were presented as the mean values.

2.4. Determination of Antioxidant Capacity

2.4.1. *DPPH (2,2-Diphenyl-1-Picrylhydrazyl) assay*

The DPPH free radical scavenging activity was assessed using a modified method from (Takao *et al*., 1994), with adaptations from (Kumarasamy *et al*., 2007). DPPH (8 mg) was dissolved in methanol (100 mL) to create an 80 µg/mL solution. Plant extract stock solutions (1 mg/mL) were serially diluted. Each diluted extract (2 mL) was mixed with 2 mL of the DPPH solution and incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a UV spectrophotometer.

The DPPH scavenging activity was calculated as follows:

DPPH scavenging activity (% inhibition) = [(A_control - A_sample) / A_control] x 100.

The IC_{50} value, representing the concentration of extract required for a 50% reduction in DPPH concentration, was determined by plotting a concentration curve and performing linear regression analysis. A lower IC_{50} indicates a higher antioxidant capacity. As a reference standard, Butylated Hydroxy Toluene (BHT) was used. All measurements were performed in triplicate, and the IC_{50} value was expressed in µg/mL.

2.5. Determination of Ferrous Ion Chelating Capacity

The ferrous ion chelating capacity of the extract was determined according to the method mentioned in (Dinis *et al*., 1994). Varying concentrations of the extract were mixed with 135 µL of the solvent. 2 mM $FeCl_2$ was added to the solution and incubated for 5 min. After that, five mM ferrozine solution was added and lasted 10 min. After incubation, absorbance was measured at 562 nm by using a spectrophotometer (Thermo Scientific Varioskan Flash) against a blank. Ethylenediaminetetraacetic acid (EDTA) was used as a reference standard. All measurements were performed in triplicate, and the IC_{50} value was expressed in mg/mL.

2.6. Antimicrobial Activity

The Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentrations (MBC), and Minimum Fungicidal Concentrations (MFC) of *E. rigida* aerial parts methanol

extract were determined using sterile 96-well plates according to the Clinical and Laboratory Standards Institute (CLSI) reference methods for bacteria (M7-A7, CLSI, 2018) and yeasts (M27-A3, CLSI, 2008). The test microorganisms included *Staphylococcus aureus* ATCC 25923, *Bacillus cereus*, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. Standard antimicrobial agents, including Chloramphenicol, Ampicillin, and Ketoconazole, were used as controls for bacteria and fungi, respectively. The analyses were conducted in triplicate, and the IC_{50} value was expressed in mg/mL.

2.7. GC-MS Analysis

For gas chromatography-mass spectrometry (GC-MS) analysis following the methodology outlined by (Aytar, 2024). Following this, the samples underwent centrifugation at 3500 revolutions per minute for 10 minutes, and the resulting supernatant was utilised for GC-MS analysis. The GC-MS analysis was conducted using the NIST Standard Reference Database protocol.

2.8. Molecular Docking Studies

Molecules were drawn in the Chem-Draw Ultra 18.0 program, and their minimal energy forms were obtained in the Chem 3D 18.0 program and saved in Mol2 format. The Protein Data Bank was used to record the enzymes (PDB). Phosphatidylinositol-specific phospholipase c (*B.cereus*) (1PTD) (2.60 Å) was chosen and preserved in PDB format. Molecule-enzyme interactions using AutoDock Vina 1.5.7 software and binding energies (kcal/mol) were determined (Trott & Olson, 2010). 2D and 3D visuals are demonstrated by BIOVIA Discovery Studio Visualizer software (Biova, 2019).

2.9. Prediction of Toxicity of Chemicals

The chemical toxicity prediction properties of selected phytocompounds have been evaluated. The 3D structures of two phytocompounds (3,5-Di-tert-butylphenol and 9-Octadecene, (E)-) were saved in PROTOX-II (https://tox-new.charite.de/protox_II/) web servers (Charite University of Medicine, Institute for Physiology, Structural Bioinformatics Group, Berlin, Germany) (Banerjee *et al*., 2018; Salaria *et al*., 2020; Rolta, Salaria, *et al*., 2021; Rolta, Yadav, *et al*., 2021).

2.10. Statistical Analysis

Correlation coefficients (R) were calculated using the CORREL statistical function in MS Excel software to determine the relationship between two variables. Data are expressed as mean ± SD obtained from three separate observations.

3. FINDINGS

In our study, the DPPH assay IC_{50} value of *E. rigida* methanol aerial parts was measured at 919.46 ± 22.51 µg/mL, while the positive control, BHT, showed an IC_{50} value of 230 ± 10 µg/mL; these results indicate that *E. rigida* has good antioxidant activity compared to BHT in terms of DPPH radical scavenging activity. Additionally, the iron chelating IC_{50} value of *E. rigida* methanol aerial parts was measured as 4.24 ± 0.04 mg/mL, while the positive control, EDTA, showed an IC_{50} value of 25.30 ± 4.44 mg/mL; these results indicate that *E. rigida* has stronger iron chelating activity compared to EDTA. *E. rigida* methanol aerial parts contain a total phenolic content of 11.96 mg GAE/g extract DW and a total flavonoid content of 26.83 mg QE/g extract DW (Table 1).

Table 1. DPPH radical scavenging activity, iron chelating activity (IC_{50} (µg/mL) ± *SD*) and total flavonoid content of *E. rigida* aerial parts methanol extract ± *SD** values.

Plant Name	DPPH (IC_{50} µg/mL)	Iron Chelating (IC_{50} mg/mL)	Total Phenolic Compound (mg GAE/g extract DW)	Total Flavonoid Compound (mg QE/g extract DW)
E. rigida methanol aerial parts extract	919.46 ± 22.51	4.24 ± 0.04	11.96 ± 1.10	26.83 ± 3.92
BHT (positive control)	230 ± 10			
EDTA (positive control)		5.30 ± 4.44		

In this study, the antimicrobial activities of *E. rigida* aerial parts methanol extract were compared with standard drugs such as Ampicillin (Amp), Chloramphenicol (C), and Ketoconazole (Keto). The MIC and MBC values of the *E. rigida* aerial parts methanol extract against the tested bacteria are presented in Table 2. The results indicate that the MIC and MBC values range from 12.5 mg/mL to >50 mg/mL. According to the antimicrobial screening results, the MIC values of *E. rigida* aerial parts methanol extract were found to be between 62.5 mg/mL and >125 mg/mL for Ampicillin, between 15.63 mg/mL and >125 mg/mL for Chloramphenicol, and 31.25 mg/mL for Ketoconazole. The methanol extract of *E. rigida* aerial parts demonstrates strong antibacterial effects against microorganisms such as *E. coli* and *B. cereus*, with MIC and MBC values of 25 mg/mL for *E. coli* and a MIC value of 12.5 mg/mL for *B. cereus*. However, the extract shows lower efficacy against pathogens such as *S. aureus* and *P. aeruginosa*. Additionally, while it exhibits some antifungal activity against *C. albicans*, its effectiveness is lower than Ketoconazole.

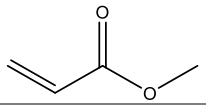
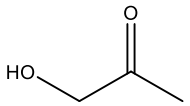
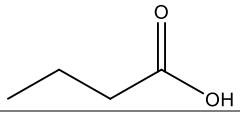
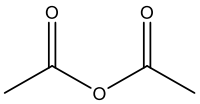
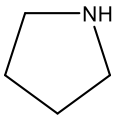
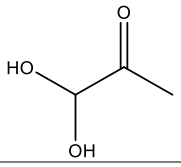
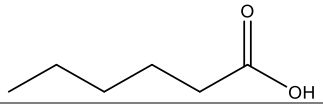
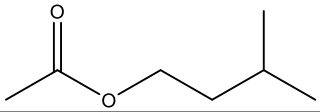
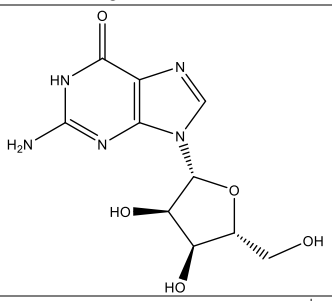
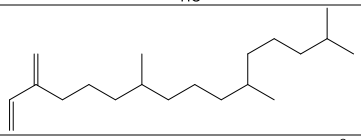
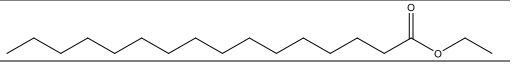
Table 2. MIC, MBC and MFC of the *E. rigida* aerial parts methanol extract and controls (mg/mL).

Microorganisms		Plant Species	Positive Control		
		Euphorbia rigida aerial parts methanol extract	Amp	C	Keto
E. coli ATCC 25922	MIC	25	>125	125	NS
	MBC	25	>125	>125	NS
S. aureus ATCC 25923	MIC	50	62.5	125	NS
	MBC	>50	62.5	>125	NS
B. cereus	MIC	12.5	125	15.63	NS
	MBC	>50	125	15.63	NS
P. aeruginosa ATCC 27853	MIC	50	>125	125	NS
	MBC	>50	>125	>125	NS
C. albicans ATCC 10231	MIC	25	NS	NS	31.25
	MFC	>50	NS	NS	62.5

Amp: Ampicillin; C: Chloramphenicol; Keto: Ketoconazole; NS: Not Studied

The methanol extract of *E. rigida* aerial parts identified various bioactive compounds. The extracts contain 11 bioactive phytochemical compounds with their retention time (RT), concentration (% area), and chemical structure presented in Table 3. The table above presents data from a compound mixture's gas chromatography (GC) analysis. This study analysed the percentage areas of compounds identified in the plant extract. Guanosine was the most abundant compound, accounting for 35.78% of the total area. Dihydroxyacetone followed this at 8.21% and Neophytadiene at 7.75%. Additionally, Pyrrolidine was detected at 4.91%, Hexadecanoic acid, ethyl ester at 4.05%, 1-Butanol, 3-methyl-, acetate at 3.49%, 2-Propenoic acid, methyl ester at 3.13%, Acetic oxide at 2.54%, n-Hexanoic acid at 2.46%, Butanoic acid at 2.43%, and Acetol at 2.48%. These results suggest that Guanosine is the dominant compound in the extract, with other compounds present in notable proportions.

Table 3. The GC-MS analysis results of *E. rigida* aerial parts methanol extract.

No	Retention time (minutes)	Compound Name	Area (%)	Molecular Structure
1	3.508	2-Propenoic acid, methyl ester	3.13	
2	4.163	Acetol	2.48	
3	4.492	Butanoic acid	2.43	
4	4.677	Acetic oxide	2.54	
5	5.098	Pyrrolidine	4.91	
6	7.512	Dihydroxyacetone	8.21	
7	10.660	n-Hexanoic acid	2.46	
8	14.381	1-Butanol, 3-methyl-, acetate	3.49	
9	24.750	Guanosine	35.78	
10	34.503	Neophytadiene	7.75	
11	37.712	Hexadecanoic acid, ethyl ester	4.05	

In this study, the docking analysis of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* (PDB ID: 1PTD) with three different ligands Guanosine, Neophytadiene, and Dihydroxyacetone was conducted to predict their binding interactions and energies (Table 4 and Figure 4).

Table 4. Docking scores and report of predicted interactions of docked conformations phosphatidylinositol-specific phospholipase c (*B.cereus*) (1PTD).

Ligand	Protein	Binding Energy (kcal/mol)	Amino acid	Interacting	Distance
Guanosine	1PTD	-5.0	A: ARG69:HH11 -: [001: O1	Conventional Hydrogen Bond	2.65
			A: ARG69:HH11 -: [001: O4	Conventional Hydrogen Bond	2.43
			A: ARG163:HH21 -: [001: O2	Conventional Hydrogen Bond	1.78
			A: SER236: HG -: [001: N2	Conventional Hydrogen Bond	2.70
			: [001:H1 - A: ASP198:OD2	Conventional Hydrogen Bond	2.26
			: [001:H2 - A: ASP198:OD2	Conventional Hydrogen Bond	1.86
			: [001:H3 - A: ASP198:OD1	Conventional Hydrogen Bond	2.02
			: [001:H4 - A: SER234:O	Conventional Hydrogen Bond	2.57
			: [001:H4 - A: SER234: OG	Conventional Hydrogen Bond	2.76
			: [001:H5 - A: SER234:O	Conventional Hydrogen Bond	1.83
			: [001:H5 - A: SER234: OG	Conventional Hydrogen Bond	2.53
			: [001:H13 - A: GLU117:OE1	Conventional Hydrogen Bond	2.36
			A: SER236:HB1 -: [001: O5	Carbon Hydrogen Bond	2.02
			A: TYR200 -: [001	Pi-Pi Stacked	5.06
			: [001 - A: TYR200	Pi-Pi Stacked	4.57
Neophytadiene	1PTD	-4.5	A: ARG163 -: [001	Alkyl	4.68
			: [001:C11 - A: ARG163	Alkyl	3.46
			: [001:C12 - A: LYS115	Alkyl	3.92
			: [001:C12 - A: ARG163	Alkyl	3.61
			: [001:C20 - A: LYS115	Alkyl	5.46
			A: HIS32 -: [001:C17	Pi-Alkyl	4.41
			A: TRP178 -: [001	Pi-Alkyl	4.38
			A: TRP178 -: [001	Pi-Alkyl	4.52
			A: TRP178 -: [001:C11	Pi-Alkyl	4.32
			A: TRP178 -: [001:C11	Pi-Alkyl	4.42
			A: TYR200 -: [001	Pi-Alkyl	4.28
			A: TYR200 -:[001:C17	Pi-Alkyl	4.74
Dihydroxyacetone	1PTD	-3.8	A: ARG69:HH22 -: [001: O3	Conventional Hydrogen Bond	1.86
			: [001:H6 - A: ASP67:OD2	Conventional Hydrogen Bond	2.70
			: [001:H5 - A: ASP198:OD1	Carbon Hydrogen Bond	2.46

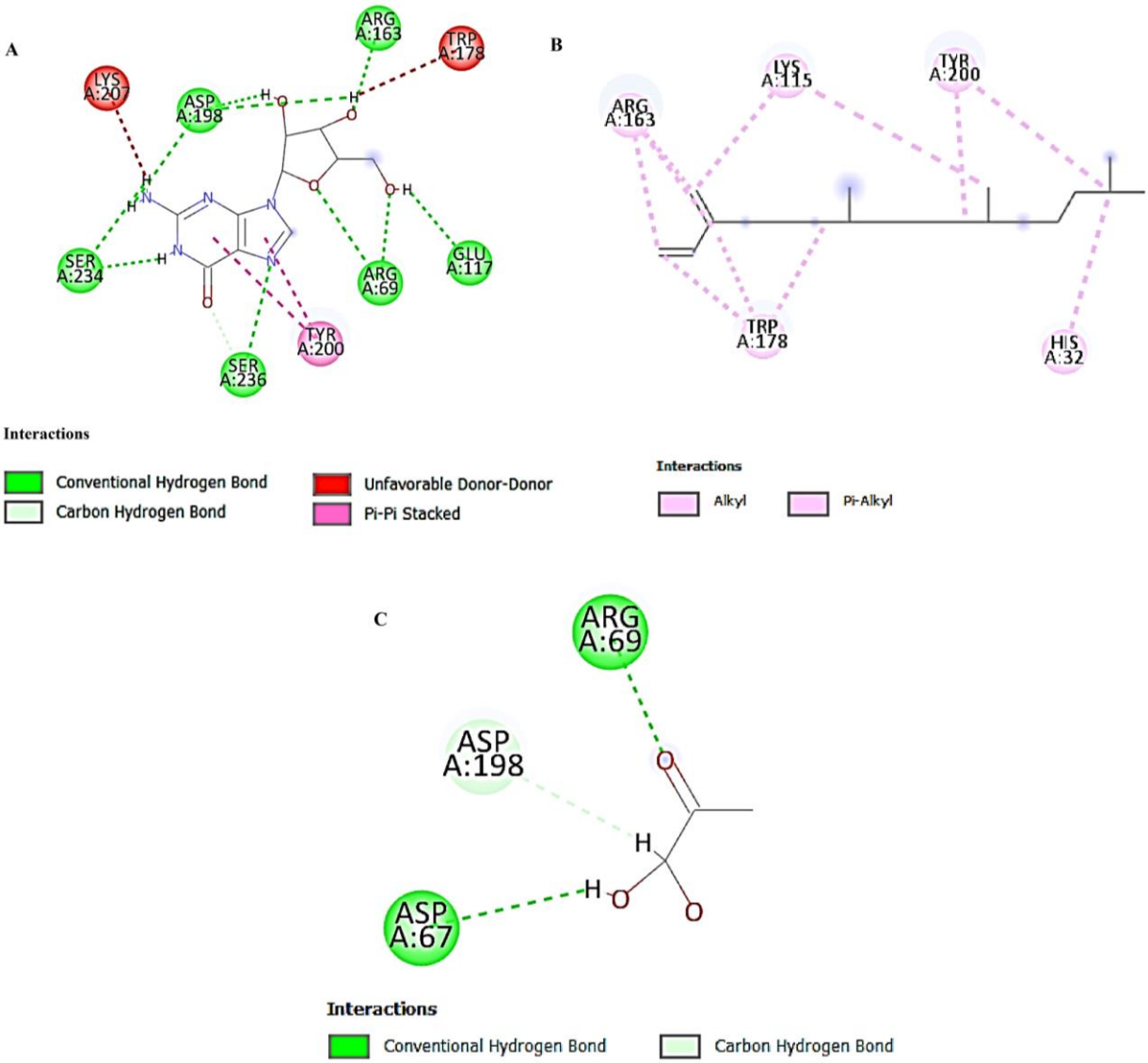


Figure 1. Molecular docking process of A) Guanosine B) Neophytadiene C) Dihydroxyacetone.

Guanosine exhibited the strongest binding affinity, with a binding energy of -5.0 kcal/mol. It formed multiple conventional hydrogen bonds with amino acid residues such as ARG69, ARG163, SER236, and ASP198, along with carbon-hydrogen bonds and Pi-Pi stacked interactions involving TYR200. These interactions suggest a stable binding conformation within the protein's active site (Figure 1).

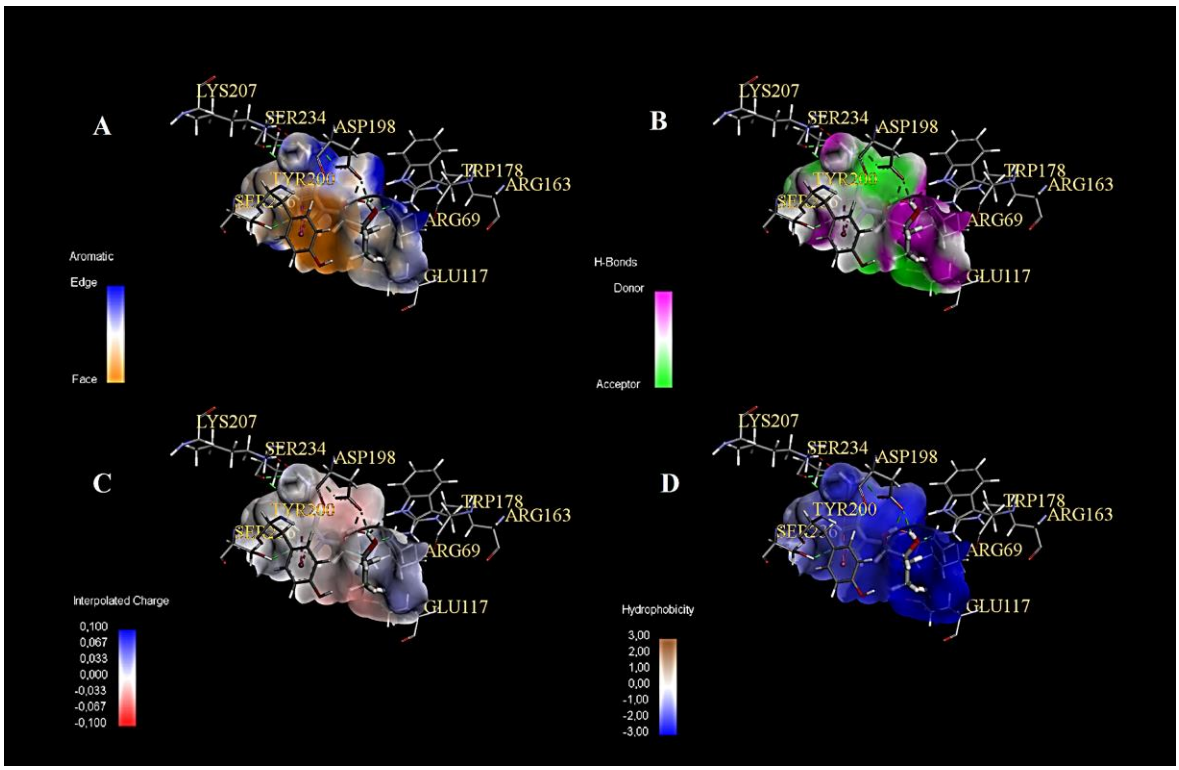


Figure 2. Interaction diagram of Guanosine with phosphatidylinositol-specific phospholipase c (*B. cereus*) (1PTD) in terms of A) Aromatic B) H- bonds C) Interpolated charge D) Hydrophobicity.

Neophytadiene, with a binding energy of -4.5 kcal/mol, primarily interacted through hydrophobic interactions such as alkyl and Pi-alkyl bonds with residues including ARG163, LYS115, HIS32, TRP178, and TYR200. These interactions indicate the ligand's potential to occupy the binding site, though with slightly lower affinity than Guanosine (Figure 2).

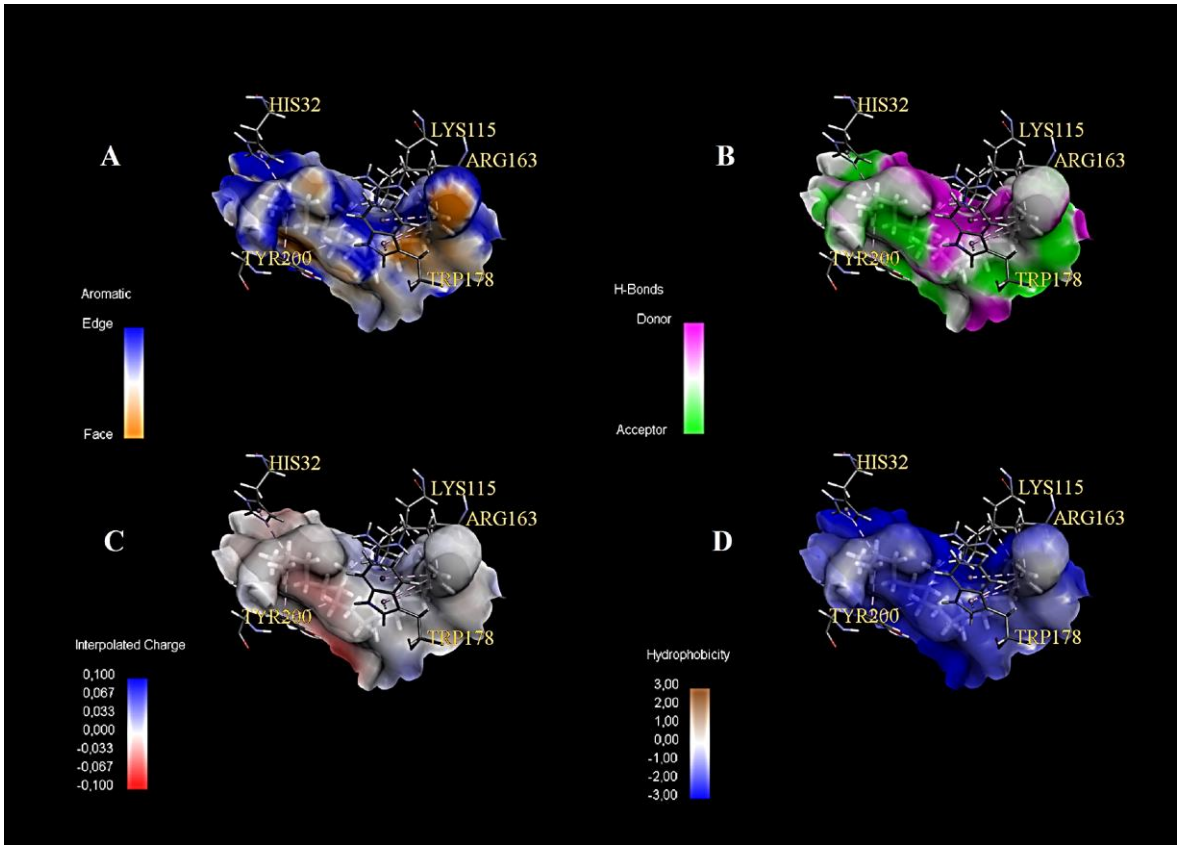


Figure 3. Interaction diagram of Neophytadiene with phosphatidylinositol-specific phospholipase c (*B. cereus*) (1PTD) in terms of A) Aromatic B) H- bonds C) Interpolated charge D) Hydrophobicity

Dihydroxyacetone showed the weakest binding energy at -3.8 kcal/mol. It formed fewer interactions, mainly conventional hydrogen bonds with ARG69 and ASP67 and a carbon-hydrogen bond with ASP198. The lower number of interactions correlates with its relatively reduced binding affinity, suggesting it may not be as effective in occupying the enzyme's active site compared to the other ligands (Figure 3).

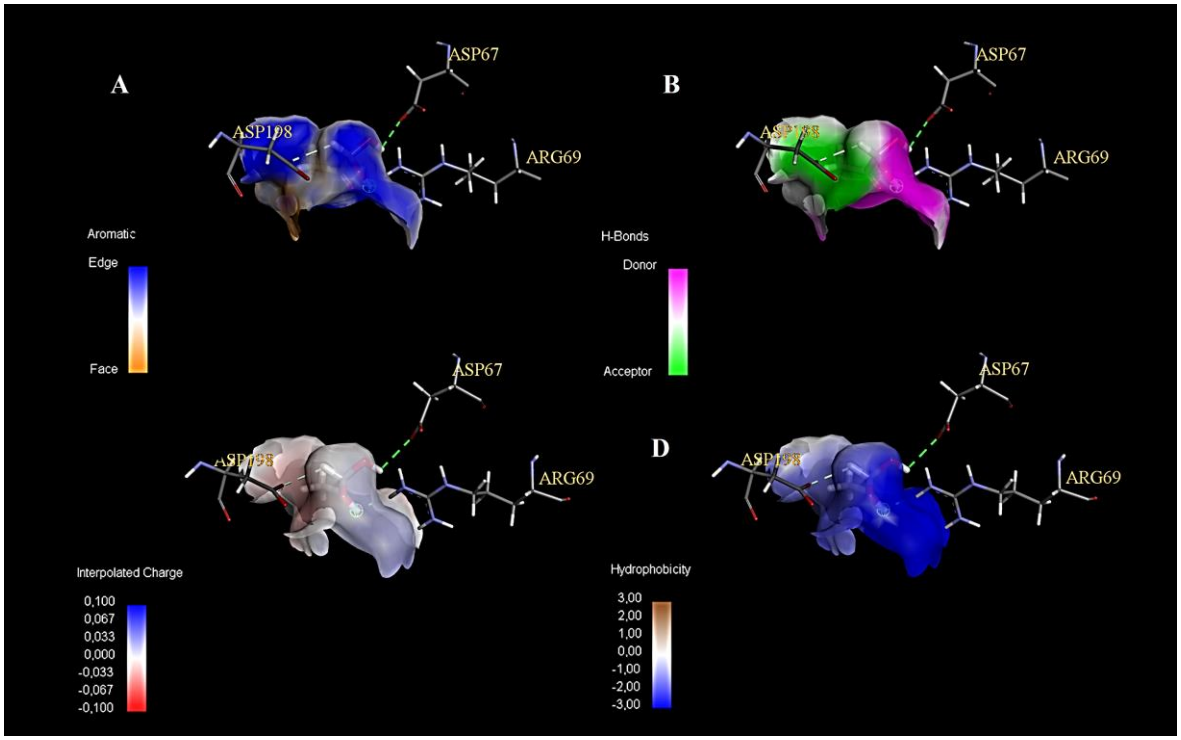


Figure 4. Interaction diagram of Dihydroxyacetone with phosphatidylinositol-specific phospholipase c (*B. cereus*) (1PTD) in terms of A) Aromatic B) H- bonds C) Interpolated charge D) Hydrophobicity

Overall, the results highlight the three ligands' varying binding affinities and interaction profiles, with Guanosine demonstrating the strongest and most diverse interactions with the phospholipase C enzyme.

The acute toxicity profile of the phytochemical components in *E. rigida* extract was extensively analysed, revealing that most of the components exhibit generally inactive toxicity effects (Table 5 and Figure 5). The LD50 values for Dihydroxyacetone, Guanosine, and Neophytadiene were determined to be 2200 mg/kg, 13 mg/kg, and 500 mg/kg, respectively. Correspondingly, the toxicity classifications for Dihydroxyacetone, Guanosine, and Neophytadiene were categorised as class 5, class 2, and class 6, indicating their varied toxicological profiles. When considering biological parameters such as hepatotoxicity, neurotoxicity, immunotoxicity, and cytotoxicity, it was observed that most components displayed inactive profiles. However, Guanosine was identified as active in both neurotoxicity and clinical toxicity assessments, suggesting that this compound may have potential toxic effects that warrant careful evaluation.

Additionally, Guanosine was found to be active in the nrf2/ARE activation and Cytochrome CYP2C9 enzymatic activity, indicating significant effects on these biochemical pathways. Overall, the results indicate that the phytochemical components of *E. rigida* extract largely do not exhibit toxic effects and generally present an inactive profile. Nonetheless, the activity of specific components suggests that further investigations are needed to assess their potential toxicological impacts and interactions thoroughly.

249 **Table 5.** Acute toxicity profile values of phytochemical components in *E. rigida* aerial parts methanol extract.

Toxicity Model	Dihydroxyacetone	Probability	Guanosine	Probability	Neophytadiene	Probability
LD_{50} (mg/kg)	2200		13		500	
Toxicity class	5		2		6	
Hepatotoxicity	Inactive	0.95	Inactive	0.73	Inactive	0.79
Neurotoxicity	Inactive	0.93	Active	0.59	Inactive	0.57
Immunotoxicity	Inactive	0.99	Inactive	0.99	Inactive	0.99
Cytotoxicity	Inactive	0.86	Inactive	0.98	Inactive	0.81
Clinical toxicity	Inactive	0.66	Active	0.57	Inactive	0.73
Androgen Receptor (AR)	Inactive	0.99	Inactive	0.99	Inactive	0.99
Estrogen Receptor Alpha (ER)	Inactive	0.98	Inactive	0.98	Inactive	0.98
Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	Inactive	0.97	Inactive	0.75	Inactive	1
Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	Inactive	0.98	Inactive	0.99	Active	1
Mitochondrial Membrane Potential (MMP)	Inactive	1	Inactive	0.98	Inactive	0.99
Phosphoprotein (Tumor Suppressor) p53	Inactive	0.98	Inactive	0.82	Inactive	1
Thyroid hormone receptor alpha (THRα)	Inactive	0.90	Inactive	0.90	Inactive	0.90
Thyroid hormone receptor beta (THRβ)	Inactive	0.78	Inactive	0.78	Inactive	0.78
GABA receptor (GABAR)	Inactive	0.96	Inactive	0.96	Inactive	0.96
Achetylcholinesterase (AChE)	Inactive	0.95	Inactive	0.73	Inactive	0.79
Pregnane X receptor (PXR)	Inactive	0.92	Inactive	0.92	Inactive	0.92
Voltage-gated sodium channel (VGSC)	Inactive	0.95	Inactive	0.95	Inactive	0.95
Cytochrome CYP1A2	Inactive	0.95	Inactive	0.99	Inactive	0.94
Cytochrome CYP2C19	Inactive	0.93	Inactive	0.88	Inactive	0.94
Cytochrome CYP2C9	Inactive	0.68	Inactive	0.75	Active	0.71
Cytochrome CYP2D6	Inactive	0.90	Inactive	0.92	Inactive	0.77
Cytochrome CYP3A4	Inactive	0.98	Inactive	0.99	Inactive	0.99
Cytochrome CYP2E1	Inactive	0.95	Inactive	0.98	Inactive	0.96

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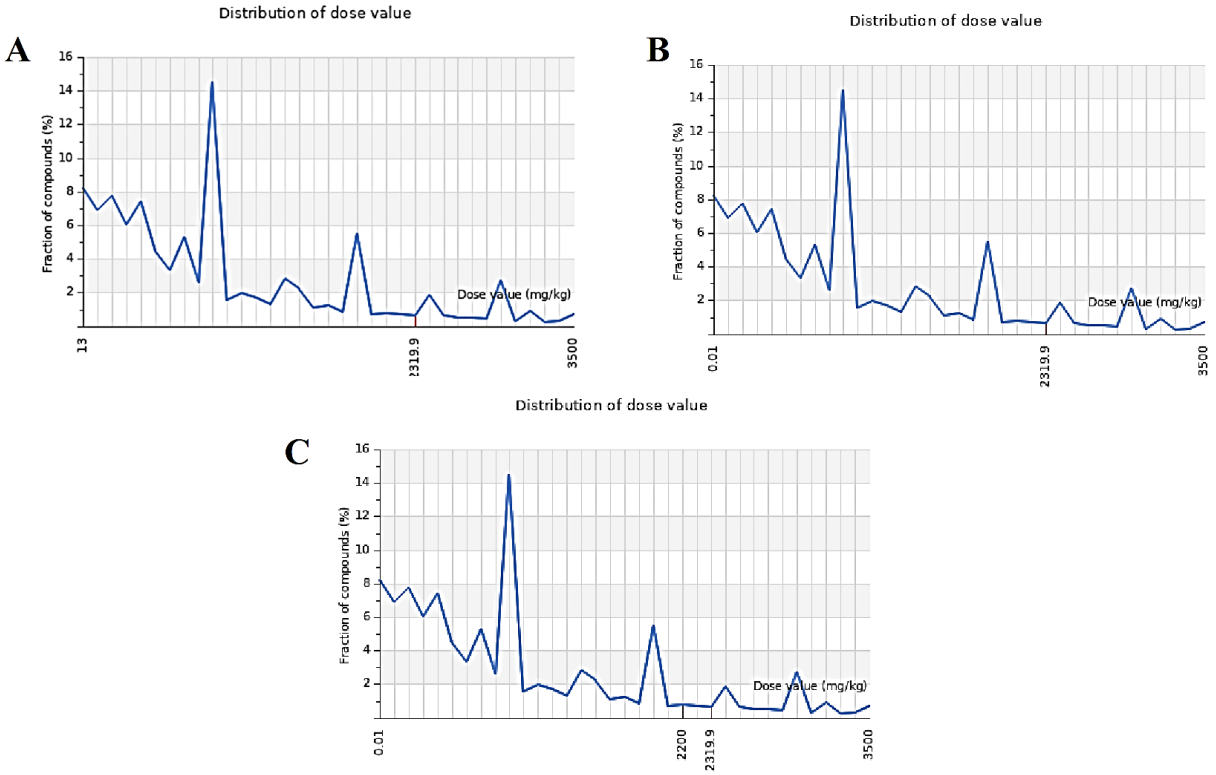

Figure 5. Acute Toxicity Profiles in *E. rigida* aerial parts methanol extract A) Guanosine B) Neophytadiene C) Dihydroxyacetone.

4. DISCUSSION and CONCLUSION

The pharmacological potential of Euphorbia species has attracted considerable attention, primarily due to their diverse bioactive properties. Studies on these plants have examined their antioxidant, antibacterial, and antifungal activities, highlighting potential applications in medical and therapeutic fields. Key parameters such as phenolic content, DPPH radical scavenging activity, iron chelating capacity, flavonoid content, and antimicrobial properties are crucial for assessing the biological activities of *Euphorbia* species.

Our study comprehensively evaluates *Euphorbia* species regarding phenolic content, DPPH radical scavenging activity, iron chelating capacity, flavonoid content, and antimicrobial activities. The detailed analyses of these parameters provide a deeper understanding of the therapeutic potential of *Euphorbia* species. Our results underscore the significance of these plants in health and medicine, establishing a fundamental reference for pharmacological investigations and enhancing the understanding of their potential therapeutic applications.

In the study by Aslantürk *et al*., (2021), the antioxidant activities of *E. rigid*a methanol extract were as follows: At a concentration of 10 mg/mL, the DPPH radical scavenging activity was 32.40% ± 0.004 and the iron chelating activity was 38.43; at 25 mg/mL, the DPPH radical scavenging activity was 34.21% and the iron chelating activity was 42.31%. At 50 mg/mL, the DPPH radical scavenging activity increased to 55.75%, and the iron chelating activity was 45.18% ± 0.017. At 100 mg/mL, the DPPH radical scavenging activity was 78.13%, and the iron chelating activity was 49.60%. At the highest 150 mg/mL concentration, the DPPH radical scavenging activity was 82.40%, and the iron chelating activity was 51.44%.

The study by Aslantürk *et al*. highlights a dose-dependent increase in antioxidant activity with higher concentrations, indicating substantial antioxidant efficacy. In contrast, our study demonstrates significant antioxidant activity with low IC_{50} values for the methanol extract of *E. rigida* aerial parts, suggesting strong antioxidant potential even at lower concentrations.

In the study by Kocazorbaz (2021), the aqueous extract of *E. rigida* leaves contained flavonoids and phenolics at concentrations of 0.086% per gram of leaf and 0.225% per gram of leaf, respectively. These findings indicate that *E. rigida* leaves are a rich phytochemical source of phenolic and flavonoid compounds. Both studies highlight the rich bioactive compound profile of *E. rigida*; however, our findings indicate higher concentrations of phenolics and flavonoids. This difference can be attributed to the variety of extraction methods and solvents used, which can significantly impact the yield of these compounds. The high phytochemical levels identified in our study suggest a strong antioxidant potential and underscore the importance of *E. rigida* for potential applications in health and medicine.

In the study by Ibraheim *et al*., (2013), the ethyl acetate fraction of *Euphorbia aphylla* demonstrated inhibitory activity ranging from 27.7% to 89.4% at concentrations from 10 µg/mL to 250 µg/mL. The alcohol fraction showed an inhibition rate of 77.7% across concentrations from 10 µg/mL to 500 µg/mL. However, the hexane and chloroform fractions exhibited no antioxidant activity at concentrations ranging from 10 µg/mL to 500 µg/mL. Compared to the ascorbic acid standard, the *E. aphylla* fractions exhibited strong antioxidant activity, demonstrating inhibition rates ranging from 47.1% to 99.6% at 10 µg/mL to 250 µg/mL concentrations. In our study, the DPPH radical scavenging activity of the methanol extract from the aerial parts of *E. rigida* was measured, yielding an IC_{50} value. Compared with the study by Ibraheim *et al*., it was found that the ethyl acetate and alcohol fractions of *E. aphylla* exhibit strong antioxidant activity. These findings indicate that both species possess significant antioxidant potential, but their effects may vary depending on specific extraction methods and measurement conditions.

In the study by Al-Ansi *et al*., (2024), the ethyl acetate extract of *E. arbuscula* stem latex exhibited the highest antioxidant activity with an IC_{50} value of 13.55 µg/mL, comparable to that of ascorbic acid, which had an IC_{50} value of 4.09 µg/mL. The chloroform extract followed with an IC_{50} value of 21.87 µg/mL, while the ethanol extract showed an IC_{50} value of 695.33 µg/mL, the butanol extract had an IC_{50} value of 195.17 µg/mL, and the methanol extract was reported to have an IC_{50} value of 463.73 µg/mL. In contrast, the methanol extract from the aerial parts of *E. rigida* demonstrated higher IC_{50} values for DPPH radical scavenging activity. In contrast, the ethyl acetate extract of *E. arbuscula* in Al-Ansi *et al*.'s study exhibited lower IC_{50} values, indicating stronger antioxidant activity. Additionally, the varying IC_{50} values of the methanol, ethanol, butanol, and chloroform extracts highlight that the antioxidant profiles of extracts obtained with different solvents vary. These findings suggest that different species within the same genus or different extracts of the same species can exhibit distinct antioxidant activities, and the choice of solvent significantly influences this activity.

In the study by Zeghad *et al*., (2016), the total phenol content of *E. biumbellata* leaves was reported as 15.13 g/100g GAE in methanol extracts, 14.33 g/100g GAE in water, 15.79 g/100g GAE in methanol-water mixture, and 12.695 g/100g GAE in ethanol. The total flavonoid content of *E. biumbellata* leaves was measured as 5.292 g/100g QE in methanol, 5.006 g/100g QE in water, 7.06 g/100g QE in the methanol-water mixture, and 3.993 g/100g QE in ethanol. For *E. dendroides* seeds, the total phenol content was found to be 3.035 g/100g GAE in methanol, 2.955 g/100g GAE in water, 3.215 g/100g GAE in methanol-water mixture, and 1.84 g/100g GAE in ethanol. The total flavonoid content of *E. dendroides* seeds was determined as 2.846 g/100g QE in methanol, 2.266 g/100g QE in water, 2.98 g/100g QE in a methanol-water mixture, and 1.926 g/100g QE in ethanol.

The methanol extract of *E. rigida* aerial parts shows significant differences in phenolic and flavonoid contents when compared with the findings from the study by Zeghad *et al*. on *E. biumbellata* and *E. dendroides*. The total phenolic content of *E. rigida* methanol extract is lower than *E. biumbellata* and *E. dendroides*, indicating that the methanol extract of *E. rigida* aerial is less rich in phenolic compounds. In contrast, E. rigida methanol extract's total flavonoid content is notably higher than that of the other species. This suggests that the methanol extract

of *E. rigida* aerial parts may be a more significant source of flavonoid compounds. In the study by Basma *et al*., (2011), the DPPH radical scavenging activity of *E. hirta* leaf extract was reported to be 72.96%. The flower extract demonstrated 52.45%, the root extract 48.59%, and the stem extract 44.42% radical scavenging activity. Total phenolic content analysis revealed that the leaf extract contained 206.17 mg GAE/g, the flower extract 117.08 mg GAE/g, the root extract 83.15 mg GAE/g, and the stem extract 65.70 mg GAE/g. Regarding total flavonoid content, the leaf extract had 37.970 mg CEQ/g, the flower extract 35.200 mg CEQ/g, the root extract 24.350 mg CEQ/g, and the stem extract 24.120 mg CEQ/g.

The study by Basma *et al*. (2011) found that the phenolic content of *E. hirta* leaf extract is significantly higher compared to the methanol extract of *E.rigida methanol* aerial parts. This finding indicates that *E. hirta* has a richer phenolic component content. Additionally, the total flavonoid content of *E. rigida* methanol extract is lower than that of *E. hirta* leaf extract but higher than the flavonoid content of *E. hirta* root and stem extracts. In our study, the DPPH radical scavenging activity of *E. hirta* leaf extract demonstrates that both *E. hirta* and *E. rigida* methanol extract from aerial parts have high antioxidant capacities.

These results highlight that different plant species within the same genus can exhibit considerable variability in their phenolic and flavonoid contents. Furthermore, the choice of extraction methods and solvents can significantly influence the extracts' antioxidant activity and component profiles. The data underscore the importance of considering the extraction methods and solvents used, as they play a crucial role in determining plant extracts' antioxidant potential and phytochemical composition. Studies have demonstrated that the methanol extracts of *E. rigida* aerial parts have notable antibacterial and antifungal properties.

In the study by Fred-Jaiyesimi & Abo (2010), the extract of *E. heterophylla* demonstrated significant activity against *S. albus*, *P. mirabilis*, *E. coli, S. typhi*, and *K. pneumoniae*. In this study, the methanol extract of *E. rigida* leaves exhibited strong antibacterial effects against microorganisms such as *E. coli* and *B. cereus*. These findings suggest that *E. rigida* extract could be an effective natural solution for combating infections and holds potential as a treatment option in the healthcare sector. Its antibacterial properties highlight its significance in discovering natural infection prevention and treatment sources.

The methanol extracts of *E. rigida* aerial parts exhibit significant antibacterial and antifungal activities. Fred-Jaiyesimi and Abo demonstrated that extracts of *Euphorbia heterophylla* show substantial efficacy against pathogens such as *S. albus, P. mirabilis, E. coli, S. typhi*, and *K. pneumoniae.* In our study, the methanol extracts of *E. rigida* aerial parts demonstrated strong antibacterial activity against *E. coli* and *B. cereus.*

In the study by Al-Ansi *et al*. (2024), the extracts and pure compounds isolated from the stem latex of *E. arbuscula* exhibited weak or no antibacterial activity against *S. aureus*, *E. coli*, and *P. aeruginosa*. In the study by Ibraheim *et al*. (2013), *E. aphylla* ethyl acetate fraction demonstrated significant antibacterial activity, with inhibition zones ranging from 12 mm to 16 mm against *E. coli, P. aeruginosa, B. cereus,* and *M. luteus*. The alcohol fraction was particularly effective against *E. coli* and P. *aeruginosa*, showing inhibition zones of 16 mm and 12 mm, respectively. The chloroform fraction exhibited moderate antibacterial activity, with 14 mm and 13 mm inhibition zones against *E. coli* and *P. aeruginosa*. No antifungal activity was observed in any of the fractions. In contrast, the standard antifungal agent clotrimazole effectively inhibited all tested fungi, with inhibition zones ranging from 18 mm to 34 mm. The standard antibiotic chloramphenicol was effective against all bacterial strains, with inhibition zones ranging from 14 mm to 28 mm. The hexane fraction did not show antimicrobial activity against either bacteria or fungi. Al-Ansi *et al*. found that extracts isolated from the stem latex of *E. arbuscula* exhibited weak antibacterial activity against *S. aureus, E. coli*, and *P. aeruginosa*. Ibraheim *et al*. reported that the ethyl acetate fraction of *E. aphylla* demonstrated significant antibacterial activity against various bacteria, although no antifungal activity was

observed. Our study highlights the robust antibacterial properties of the methanol extracts of *E. rigida* aerial parts.

In the study by Hussain *et al*. (2014), ethanol and methanol extracts of *E. hirta* produced inhibition zones with diameters of 21.15 mm, 22.85 mm, 22.80 mm, and 23.55 mm against *S. aureus*, *E. coli*, and *C. albicans*, respectively. In contrast, ethanol and methanol extracts of *E. thymifolia* exhibited smaller inhibition zones for the same microorganisms, with diameters of 16.84 mm, 18.56 mm, and 20.24 mm against *S. aureus* and 17.84 mm, 19.23 mm, and 20.65 mm against *E. coli*. For C. *albicans, E. hirta* extracts produced inhibition zones of 16.15 mm and 17.90 mm, while *E. thymifolia* extracts yielded zones of 11.49 mm, 12.85 mm, and 14.64 mm. Hussain *et al*. found that the ethanol and methanol extracts of *E. hirta* produced significant inhibition zones against *S. aureus*, *E. coli*, and *C. albicans*. In our research, the methanol extract of *E. rigida* aerial parts exhibited high antibacterial activity against *E. coli* and *B. cereus* compared to standard antibiotics. However, its efficacy against other pathogens was lower, and its antifungal activity against *C. albicans* was less effective than Ketoconazole.

The findings of this study highlight the significant antioxidant and antimicrobial properties of the methanol extract of *E. rigida* aerial parts. The extract demonstrates notable antioxidant potential, as evidenced by its DPPH radical scavenging activity and iron chelating capacity. The total phenolic and flavonoid contents further substantiate the extract's antioxidant efficacy. Antimicrobial assays reveal that the extract exhibits potent antibacterial effects, particularly against *E. coli* and *B. cereus*. However, its effectiveness varies against other pathogens, with lower antifungal activity against *C. albicans* than standard antifungal agents such as Ketoconazole.

Molecular docking analyses indicate that Guanosine, Neophytadiene, and Dihydroxyacetone interact with phosphatidylinositol-specific phospholipase C from *B. cereus*, with Guanosine showing the strongest binding affinity. Guanosine forms a stable binding conformation within the enzyme's active site and exhibits a diverse interaction profile, suggesting its potential as a therapeutic agent. Toxicological evaluations reveal that most of the phytochemical components of *E. rigida* extract show low toxicity. However, Guanosine is identified as having significant toxicological effects, including neurotoxicity. These varied toxicity profiles necessitate further investigation to fully understand the implications of Guanosine’s effects on biological systems. Overall, *E. rigida* aerial parts show promising antioxidant and antimicrobial potential. However, the toxicological concerns, particularly about Guanosine, highlight the need for further research to ensure safety and efficacy for potential therapeutic applications.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Emine İncilay Torunoğlu: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Turan Akdağ:** Supervision, and Validation. Authors may edit this part based on their case.

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Research Article

Biological activities of silver nanoparticles synthesized using *Olea europaea* L. leaves

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Abstract: Recent advancements in nanotechnology have led to an increased utilization of silver nanoparticles (AgNPs) across various domains, including health, medicine, environmental chemistry, nanobiotechnology, and biosensors. The primary focus of this study is the green synthesis of AgNPs utilizing *Olea europaea* L. leaves. AgNP was characterized through UV-Vis Spectroscopy, SEM, EDS, and TEM. Furthermore, the study explored the antimicrobial, antibiofilm, and antioxidant activities, along with the growth kinetics of *Staphylococcus aureus* ATCC 25923, for the synthesized AgNPs. Characterization tests confirmed the synthesis of spherical nanoparticles with a size ranging from 51 to 56 nm. AgNPs demonstrated effectiveness, particularly against *Acinetobacter baumannii* ATCC 19606 and *Proteus vulgaris* ATCC 13315 bacteria, in terms of antimicrobial and antibiofilm activities. Moreover, the AgNPs exhibited noteworthy antioxidant activity. This study provides evidence that this environmentally friendly and cost-effective method can be applied for large-scale AgNP synthesis.

1. INTRODUCTION

Nanotechnology research has gained global prominence and is employed in numerous products, ranging from sunscreens, cosmetics, textiles, and sports equipment to applications in drug delivery, plant disease prevention, environmental pollutant remediation, biosensors, and various biomedical applications (Boisseau & Loubaton, 2011). The advancement in nanotechnology prompts consideration of eco-friendly technologies for selecting and producing nanoparticles tailored to specific purposes. Silver nanoparticles (AgNPs) hold a prominent position, exhibiting excellent antagonistic properties, sterilant capabilities, as well as pharmacological features such as biofilm removal, antioxidant effects, cell-level degradation, and anticancer effects. Plant-based sources prove to be efficient reducing agents in the synthesis of AgNPs (De Matteis *et al*., 2019). Plant nanoparticle synthesis is gaining importance as an environmentally friendly and sustainable method. As an alternative to traditional chemical synthesis techniques, nanoparticles derived from plants offer biocompatible and non-toxic compounds (Alsulami *et al*., 2023). Plants naturally contain a wealth of biochemical compounds, enabling high efficiency and activity in nanoparticle synthesis. Additionally,

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utilizing plant sources helps reduce the use of toxic chemicals, contributing to the protection of ecosystems (Piro *et al*., 2023). These nanoparticles have significant application potential in various fields, including agriculture, medicine, food safety, and environmental engineering, providing safer solutions for health and the environment while making important contributions to the advancement of scientific research and industrial applications. Therefore, plant nanoparticle synthesis holds substantial significance both scientifically and practically (Rahimzadeh *et al*., 2022). Nanoparticle synthesis through plants occurs based on natural biochemical processes and interactions of plant metabolites. This process typically begins with the combination of plant extracts, derived from various parts such as leaves, roots, or fruits, with metal salts. Plants possess the ability to reduce and stabilize metal ions due to their rich content of phenolic compounds, flavonoids, and other biologically active substances. This reduction process facilitates the transformation of metal ions into nanoparticles at the nanoscale. Furthermore, plant compounds can influence the surface properties of the resulting nanoparticles, enhancing their stability and reactivity. Consequently, this biological method provides an environmentally friendly and sustainable approach to nanoparticle production, contributing to the reduction of harmful waste associated with traditional chemical methods. This mechanism enables the use of plants' natural capabilities to produce high-quality nanoparticles with significant application potential across various fields (Barzinjy *et al*., 2020).

Olea europaea L., a prominent member of the Oleaceae family comprising approximately 29 genera and 600 species, holds great importance (Özcan & Matthäus, 2017). The leaves and fruits of *O. europaea* are the most commonly utilized parts of this plant (Alesci et al., 2022). Among the bioactive compounds present in *O. europaea* are phenolic compounds, carotenoids, secoiridoids, and flavonoids. Oleuropein is the primary phenolic compound in olives, while other notable phenolic compounds include hydroxytyrosol, verbascoside, luteolin-7-glucoside, and apigenin-7-glucoside (Bonvino *et al*., 2018). *Olea europaea* is of considerable medical significance and has a historical association with various health benefits, including cardiovascular, antioxidant and anti-inflammatory properties, anticancer, anti-diabetic effects, antimicrobial, and positive effects on skin health (Salık & Çakmakçı, 2021). *O. europaea* has great potential for nanoparticle synthesis due to its high phenolic compound content and various biological activities. Consequently, there is a need to explore the use of waste materials with high phenolic compound content, such as olive leaf (OL), in these processes.

The objective of this study was twofold: to transform a significant waste product of the olive tree into a value-added product and to unveil the biological activities of the resultant nanotechnological product. To achieve this goal, AgNPs were synthesized using olive leaf extract (OLE) through a straightforward, cost-effective, and environmentally friendly green synthesis method. The nanoparticles were characterized, and its antimicrobial, antibiofilm, antioxidant and growth kinetic effects were determined.

2. MATERIAL and METHODS

2.1. Preparation of OLE and Synthesis of AgNPs

OLs collected from Çanakkale were washed and dried before being boiled in 100 mL of sterile distilled water for 7 min. The prepared OLE was subjected to a reaction with $AgNO_3$ at 25°C. The reaction proceeded until the anticipated development of a dark color, signifying the reduction of silver ions to a brown hue. After centrifugation (10.000 rpm, 5 min.), the liquid phase was removed, and the remaining solid phase was washed several times with pure water. The resulting AgNPs were then dried in an oven (at 65°C for 48 hr) (Bayğu, 2020).

2.2. Characterization of AgNPs

The morphology, size, and chemical analysis of the AgNPs were determined using a dual-beam spectrophotometer (UV-VIS, 200- 800 nm) Scanning Electron Microscope (SEM), Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray Spectroscopy (EDS).

2.3. Antimicrobial and Antibiofilm Activities

The antimicrobial activities of OLE and AgNPs were determined using disk diffusion and microdilution methods against 8 test cultures (*Acinetobacter baumanii* ATCC 19606, *Escherichia coli NRRLB 3704*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Staphylococcus haemolyticus* ATCC 43252, *Candida albicans* ATCC 10231) (CLSI, 2006). By utilizing the findings of minimum inhibitory concentration (MIC) results, the minimum bactericidal concentrations (MBC) were also determined. The analyses were performed in triplicate.

The antibiofilm activities AgNP was conducted according to O'Toole (2011). AgNPs were utilized at MIC and MIC/2 concentrations. The percentage of biofilm inhibition is calculated using the formula:

$$\% \text{ Inhibition} = [1 - (OD_{620} \text{ of cells treated with AgNPs} / OD_{620} \text{ of negative control})] \times 100$$

2.4. Determining the Effect of AgNPs on The Growth Kinetics of *S. aureus*

S. aureus was incubated with AgNP at different concentrations (6.25 and 3.13 µg/mL), and absorbance measurements were taken at 540 nm at various time intervals (2-14 hours) to assess the effect on bacterial growth. (Erci, 2018). The analyses were performed in triplicate.

2.5. Antioxidant Activity

2.5.1. *DPPH radical scavenging activity*

The determination of DPPH was carried out according to the method described by Blois (1958). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was utilized, and the absorbance value was measured at 517 nm. The study standard was butylated hydroxytoluene (BHT). The absorbance values (A) were evaluated relative to the control. The percentage of inhibition is calculated using the formula: $\%\text{Inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100$

2.5.2. *$ABTS^{\bullet+}$ radical cation scavenging activity*

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical cation scavenging activity assay was determined according to the Blois (1958). The inhibition was calculated for the obtained values and these results were evaluated by comparing them with the positive control (BHT).

$ABTS^{\bullet+}$ Radical Cation Scavenging Activity Inhibition (%): $[(A_0 - A_1)/A_0] \times 100$

A_0: $ABTS^{\bullet+}$ of control, A_1: $ABTS^{\bullet+}$ of sample.

3. RESULTS and DISCUSSIONS

The synthesis of AgNPs from OL occurred at room temperature and was detected through a color change from yellow to brown within 2 hours (Figure 1). SEM and TEM studies confirmed the development of NPs with almost the same spherical in 4 shapes and dimensions particle size ranged between 51 to 56 nm (Figure 2A-B).

In EDS analysis, the peak at 3 keV is attributed to silver 1 atoms (Figure 2C), while other peaks are identified as chlorine (Cl) and oxygen. These peaks are reported to originate from phenolic and flavonoid components found in the oil (Veisi *et al*., 2019). The UV-Vis spectra of OL and AgNPs reveal distinct peaks at 372 and 449 nm, respectively (Figure 2D). The observed surface plasmon resonance (SPR) bands closely resemble data found in the literature for AgNPs, supporting the evidence of AgNP synthesis (Atalar *et al*., 2022). Our study's SEM and EDS analysis findings align with the literature information (Sellami *et al*., 2021; Atalar *et al*., 2022). The differences in AgNP sizes are thought to vary depending on the synthesis steps, extract concentration, and the phytochemical composition of the collected olive leaves. SEM and EDS analyses indicate that the nanoparticles obtained have both a spherical form and a size suitable for entering cells, highlighting their potential use in biological and medical studies (Erdoğan *et*

al., 2019). The morphology of the AgNPs obtained in our study is suitable for potential applications in this regard.

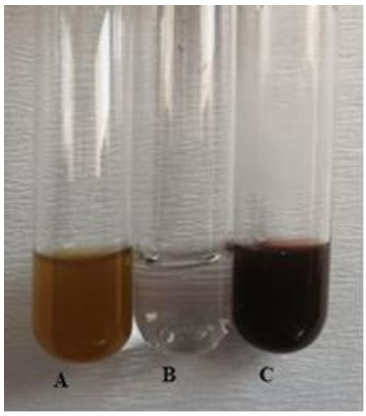


Figure 1. A) OLE, B) $AgNO_3$, C) Synthesised AgNPs.

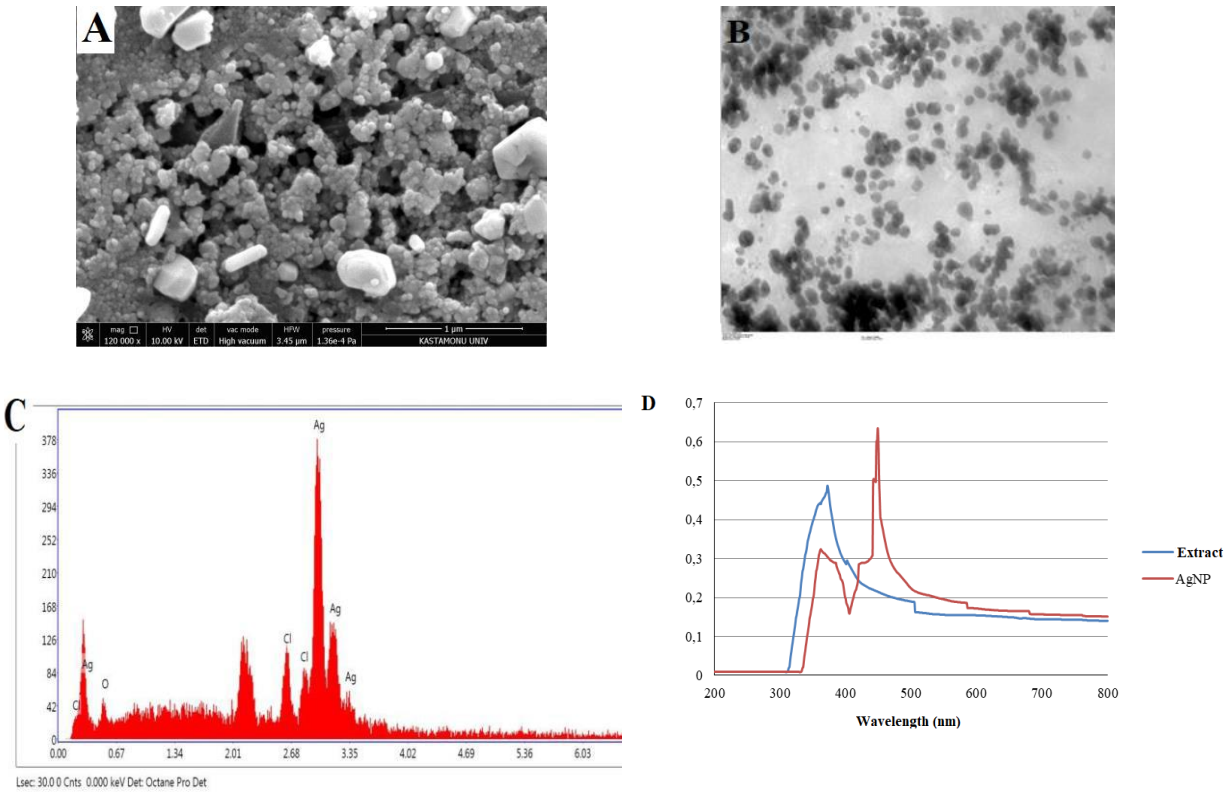


Figure 2. Characterization of AgNPs; A) SEM image, B) TEM image, C) EDS analysis, D) UV-Vis spectrum.

It was observed that OLE exhibited a higher antagonistic effect against *P. aeruginosa* and *S. haemolyticus* compared to P10. AgNP demonstrated a higher antibacterial effect than the reference antibiotic against *P. aeruginosa*, *P. vulgaris*, and *A. baumannii*. The MIC of AgNP also revealed a bacteriostatic effect higher than the S10 against *P. vulgaris* (0.625 µg/mL) and *A. baumannii* (2.5 µg/mL), consistent with the results of the disk diffusion. These findings were further supported by MBC results (Table 1).

Sellami *et al*. (2021) reported that AgNPs caused logarithmic decrease against *B. subtilis*, and some gram (-) bacteria at levels equivalent to commercial antibiotics. The study also suggested that higher MIC values against gram (-) bacteria might be due to the easier penetration of particles into the cell wall composition of gram (-) bacteria. Atalar *et al*. (2022) observed inhibitory activity against all pathogens in varying proportions ranging from 0.03 to 1.0 mg/mL of AgNPs. It is believed that the positive charge carried by Ag ions is a significant factor in the

antagonistic effect observed in interaction with microorganisms (Klueh *et al*., 2000). Therefore, the combination and synthesis of AgNPs with plant agents such as olive leaves, which possess high antimicrobial effects, may present a new strategy against microbial resistance to existing antibiotics due to the potential for high synergistic effects, as demonstrated once again in this study.

Table 1. The antimicrobial activities of OLE and AgNPs.

Test Cultures	Antimicrobial Test Methods									
	*Disc Diffusion[a]		Control (mm)		MIC (µg/mL)			MBC		
	OLE	AgNP	P10	NY100	OLE	AgNP	S10	NY100	OLE	AgNP
E. coli	9.0+0.01	8.0+0.03	16.0	NT	10.0	10.0	4.0	NT	10.0	10.0
P. aeruginosa	14.0+0.01	12.0+0.02	8.0	NT	5.0	5.0	1.0	NT	5.0	5.0
P. vulgaris	8.0+0.01	14.0+0.02	13.0	NT	1.25	0.625	4.0	NT	1.25	0.625
B. subtilis	8.0+0.01	9.0+0.01	14.0	NT	20.0	20.0	2.0	NT	20.0	20.0
S. haemolyticus	17.0+0.05	10.0+0.01	14.0	NT	1.25	10.0	4.0	NT	1.25	10.0
A. baumannii	11.0+0.01	17.0+0.01	12.0	NT	10.0	2.5	4.0	NT	10.0	2.5
S. aureus	12.0+0.012	12.0+0.05	15.0	NT	10.0	10.0	5.0	NT	10.0	10.0
C. albicans	16.0+0.03	8.0+0.04	NT	16.0	0.625	10.0	NT	2.5	1.25	10.0

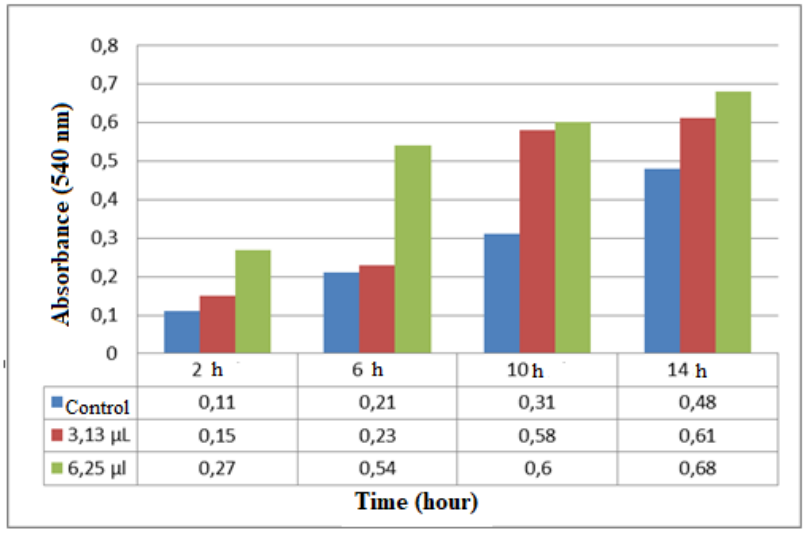
P10 = Penicillin (10 µg/disc); NY100: Nystatin (100 µg/disc); S10: Streptomycin (10 µg/disc); NT: Not Tested

Both in AgNP and OLE, the highest antibiofilm percentage was observed against *A. baumannii* (Table 2). In recent years, numerous studies have been conducted on NP synthesis from plants to develop new antibiofilm strategies (Erci, 2018; Karakaş *et al*., 2023a; Karakaş *et al*., 2023b). The results obtained in these studies have shown that nanoparticles obtained through phytosynthesis exhibit high antibiofilm effects through synergistic interactions with phytochemicals present in the composition of plants. Literature information indicates that the antibiofilm activities of AgNPs synthesized from OLE were first identified in our study. Our findings provide evidence that phytosynthesized AgNPs could serve as a potential new source for developing novel antibiofilm drugs, particularly against *A. baumannii*.

Table 2. Antibiofilm activities of OLE and AgNPs.

Test Cultures	OLE		AgNP	
	MIC	MIC/2	MIC	MIC /2
E. coli	22.75 ±0.22	28.45±0.2	32.75 ±0.22	28.45±0.20
P. aeruginosa	47.51±2.10	27.51±2.10	55.72± 0.12	-
P. vulgaris	50.40±1.60	-	57.12±0.25	-
B. subtilis	30.78±1.20	-	35.12± 0.10	-
S. haemolyticus	46.12±0.11	-	60.19±0.06	-
A. baumannii	81.04±0.01	54.12±0.12	92.04±0.01	54.12±0.12
S. aureus	32.57±1.10	-	58.41±1.12	-
C. albicans	30.78±1.20	-	35.67±0.56	-

AgNPs, especially at a concentration of 6.25 µg/mL, slowed down the growth of *S. aureus* over time (Figure 3). Studies on the growth kinetics of *S. aureus* with AgNPs are quite limited in the literature (Erci, 2018).



	2 h	6 h	10 h	14 h
Control	0,11	0,21	0,31	0,48
3,13 µL	0,15	0,23	0,58	0,61
6,25 µl	0,27	0,54	0,6	0,68

Figure 3. The time-dependent kinetics of AgNP at two different concentrations against *S. aureus.*

Although lower compared to OLE findings, an increase in antioxidant activity dependent on concentration has been achieved in the AgNP product (Figure 4A) In the CUPRAC method, although the control group is lower compared to ascorbic acid, significant antioxidant activity has been identified in both OLE and the AgNPs (Figure 4B).

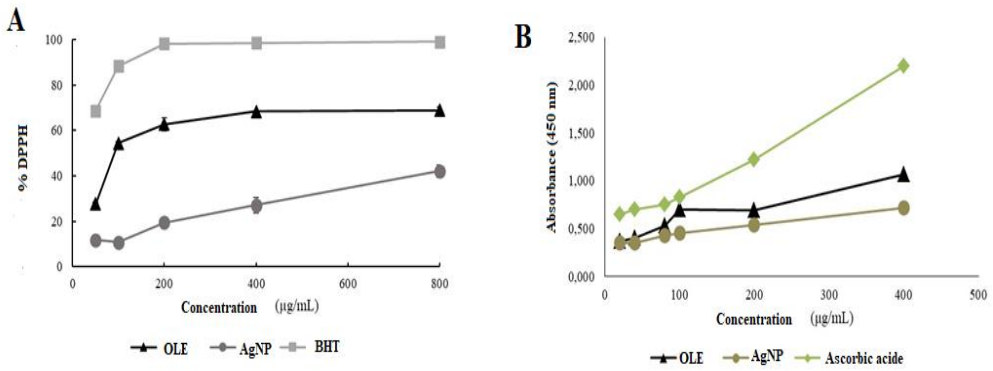


Figure 4. Antioxidant activites of extract and AgNPs. A: DPPH free radical scavenging activity, B: ABTS radical cation scavenging activity.

In the literature, while different biological activity studies on OLE-AgNP components are reported, antioxidant findings appear to be quite limited. Sellami *et al*. (2021) detected high antioxidant activity of OLE-AgNP compared to the ascorbic acid standard and OLE. They suggested that this could be attributed to metal chelation arising from electron-rich secondary metabolites (phenolics and flavonoids) derived from the plant (Khan et al., 2021; Sellami *et al*., 2021).

This study aims to highlight the potential utilization of olive leaves, a crucial component of the economically significant olive tree, as a green synthesis material in the production of AgNPs. The UV-Vis, SEM, EDS, and TEM analyses employed for characterizing the obtained AgNPs demonstrate the successful synthesis of silver nanoparticles. In addition to the morphological characteristics of the synthesized AgNPs, the antimicrobial activity was also investigated in this study, revealing particularly high activity against gram (-) bacteria (*P. vulgaris* and *A. baumannii*). Similarly, tests demonstrated a significant reduction in the biofilm formation capacity of *A. baumannii* bacteria, which is particularly important as it represents the first antibiofilm findings obtained in phytosynthesis studies with olive leaves. It was also shown that AgNPs at a concentration of 6.25 µg/mL slowed down the growth of *S. aureus*. Additionally, the obtained antioxidant activity findings of AgNPs were noteworthy. These results collectively

suggest that the synthesized AgNPs can be a potentially versatile agent for various medical purposes.

In this study, AgNP synthesis was performed using *Olea europaea* plant leaves with an eco-friendly method. These AgNPs were characterized by UV-VİS, SEM, TEM and EDS techniques and their antimicrobial, antibiofilm and antioxidant activities were investigated. Biosynthesized AgNPs showed high antimicrobial, antibiofilm and antioxidant activities compared to plant extracts and standard controls. Therefore, AgNPs synthesized from *Olea europaea* and similar plants can be combined with medicinal phytochemicals, leading to the discovery of industrial raw materials with higher activity. Our results reveal that biologically synthesized AgNPs exhibited multifunctional properties and could be used as antimicrobial and antioxidant agents.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Özge Ceylan: methodology, validation, review and editing, Nurcihan Hacıoğlu Doğru: Supervision, investigation, methodology, resources, visualization, writing—review and editing.

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Research Article

Application of HPLC for detection of sildenafil/tadalafil in marketed honey in Oman

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Abstract: Honey adulteration allegedly with phosphodiesterase-5 (PDE-5) inhibitors including sildenafil, and tadalafil is a common and dangerous practice. This study aimed to develop a procedure to detect the presence of common adulterants namely sildenafil and tadalafil using RP-HPLC. Seven commercial honey samples of local and international origin were collected from supermarkets and honey sellers. Both the adulterants in honey samples were identified and quantified with the help of an HPLC technique. Chromatographic separation was done in RP-HPLC mode using buffer: methanol: acetonitrile (5.8: 2.5: 1.7) mobile phase and diode array as a detector. The buffer used was 0.05 M Triethylamine orthophosphate pH (3.0). The results showed that four honey samples (HAD1, HAD5, HAD6, and HAD7) were adulterated with sildenafil, and among them, HAD5 contained the maximum amount of sildenafil as 22.65 mg/g of the honey sample. However, only 2 honey samples HAD4 and HAD6 were found to be adulterated with tadalafil (1.248 and 0.7 mg/g) of the tested honey sample. The result of this study warrants rigorous quality control of the commercially available honey products in Oman by the authorities. The consumption of adulterated honey samples may impact the health of consumers hence further detailed studies must be carried out to confirm the findings of the current study and novel analytical methods be developed to detect the level of other possible adulterants in this valuable product.

1. INTRODUCTION

Honey, a valuable foodstuff produced by bees contains polyphenols, organic acids, vitamins, amino acids, and enzymes. The unique flavor, aroma, and enzyme activity of honey is because of the compounds present in it which also determines its quality. Honey is a valuable traditional natural source of highly nutritive food besides possessing medicinal value. It has various pharmacological properties such as anti-inflammatory, anti-aging, antioxidant, and anticancer. European Union is the second largest producer of honey followed by China (González-Ceballos

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et al., 2023). Because honey is widely consumed owing to its nutritive and therapeutic benefits, hence it is more vulnerable to adulteration (Fakhlaei *et al*., 2020). This is achieved through two types of adulteration: direct (addition of adulterants) and indirect (mixing pure honey with low-quality, inexpensive honey or altering it through bee feeding)(Gong *et al*., 2017). Honey adulteration is effortless, economically motivated, and hard to detect. Most of the physical and chemical indices of adulterated honey are very similar to that of natural honey products (Xu *et al*., 2020; Dou *et al*., 2023). Adulterated honey is defined as any commercially marketed "pure" honey products that are found to contain more than a 5% mass ratio limit for sucrose or maltose (Ng & Reuter, 2015).

Numerous investigations showed that honey products were adulterated with natural drugs (ginseng), synthetic drugs like anti-obesity and sex stimulant agents such as sildenafil and tadalafil (Zakaria & Yacob, 2017). Tadalafil (sold under the brand name CIALIS®) and sildenafil (sold under the brand name VIAGRA®) are the two most popular phosphodiesterase 5 (PDE-5) inhibitors used to treat erectile dysfunction (ED) (FDA, 2022; Zakaria & Yacob, 2017). Erectile dysfunction (ED) a significant health issue is defined as the inability to obtain and sustain an erection strong enough for satisfying sexual performance, which has been linked to both organic and psychogenic reasons (Scaglione *et al*., 2017). Up to 60-70% of the patients suffering from ischemic heart disease (IHD) and hypertension suffer from erectile dysfunction. These PDE-5 inhibitors can interact badly with several medications used to treat patients suffering from cardiovascular diseases and thus can only be used in consultation with a licensed healthcare provider. Moreover, people with diabetes, hypertension, hypercholesterolemia, or heart disease frequently use nitrates (nitroglycerin), which may interact with these unreported substances that are used to adulterate honey and reduce blood pressure to dangerous levels (FDA, 2022). The FDA's laboratory issued a warning to four companies for selling honey-based products which upon testing were found to contain hidden active pharmaceutical ingredients including those present in the FDA-approved erectile dysfunction medications i.e., Cialis (tadalafil) and Viagra (sildenafil) (FDA, 2022). In Saudi Arabia, herbal medicines were found to contain various synthetic drugs such as sildenafil, tadalafil, and glibenclamide (Bogusz *et al*., 2006). A previous study conducted in the Sultanate of Oman showed that 7 of the 33 herbal medicines and food samples contain sildenafil, tadalafil, and vardenafil (Al Lawati *et al*., 2017).

The profit-oriented adulteration of honey is becoming a global problem. Besides being unethical practice, adulterated honey also has undeniable consequences on human health affecting kidneys, heart, brain, etc. Although the consumption of honey has increased in the last few years, the consumers' trust and interest in honey-based products due to adulteration is on the decline (Fakhlaei *et al*., 2020). Therefore, honey, a valuable food product, needs to be regularly monitored for its safety and quality. There are numerous methods available for honey adulteration detection including gas chromatography with flame ionization detector (GC-FID) or with mass spectrometry (GC-MS), high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) or tandem mass spectrometry (HPLC-MS-MS), refractive index detectors (RID), electrochemical detectors (ECD). near-infrared transflectance (NIRT) and Fourier transform infrared (FTIR) spectroscopy and many others (Cárdenas-Escudero *et al*., 2023; Mehryar & Esmaiili, 2011). These analytical techniques are modern and sophisticated and help in detecting honey adulteration but at the same time suffer from one or other limitations, i.e., cost of instrumentation, maintenance, the requirement of skilled personnel, and complicated and time-consuming. The limitation of the cost and skilled personnel was overcome with the use of high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD). The main objective of the current study is to detect sildenafil and tadalafil as possible adulterants in commercially available honey products that are claimed to boost sexual performance.

2. MATERIAL and METHODS

A total of seven honey samples were collected from different grocery stores, and supermarkets in Oman. Collected honey samples were coded as HAD1 to HAD7 to hide the identity of the manufacturers and to avoid any conflict of interest. The collection location and coding of honey samples are presented in Table 1, which shows the location from where it was collected. The honey samples were tested for the presence of sildenafil and tadalafil contents using HPLC (Agilent, 1220 Infinity II). Each experiment was repeated twice. All the chemicals including the solvents methanol, acetonitrile, and the buffers triethylamine orthophosphate were used of HPLC grade and purchased by Merck AG, Darmstadt, Germany. The standard sildenafil and tadalafil were received as gift samples from the Ministry of Health, Sultanate of Oman. The standard stock solution of 200 µg/mL of sildenafil/tadalafil in methanol was prepared.

Table 1. Code, location, and the manufactured honey samples.

S. No	Code Assigned	Location
1	HAD1	Al Seeb
2	HAD2	Al Mawaleh South
3	HAD3	Al Khuwair South
4	HAD4	Al Ghubrah
5	HAD5	Al Seeb
6	HAD6	Al Mawaleh South
7	HAD7	Al Ghubrah
Total Samples		07

2.1. Apparatus

The HPLC separation was performed on a C18 Agilent HPLC column (250 × 4.6 mm), having a particle size of 5 microns using an Agilent 1220 LC system with a diode array detector (DAD).

2.2. Reference Standard Preparation

The pure drug sample of 10 mg of sildenafil (98.8% potency) or tadalafil (99.8% potency) was dissolved in 10 mL of methanol in a volumetric flask. The solution was sonicated for 10 min and then diluted up to 50 mL with methanol for HPLC analysis.

2.3. Sample Preparation

An accurately weighed honey sample (0.5 g) was dissolved in 50 mL of methanol. The extract was centrifuged for 5 min at 4000 rpm. The supernatant was collected and filtered through a 0.45 µm filter and used as such for the HPLC analysis.

2.4. Mobile Phase Preparation

The mobile phase consisted of buffer: methanol: acetonitrile in the ratio of (5.8:2.5:1.7) was prepared by adding 580 mL of 0.05 M triethylamine orthophosphate pH (3.0) solution, 250 mL of methanol and 170 mL of acetonitrile in 1000 mL of volumetric flask. The buffer used was 0.05 M Triethylamine orthophosphate pH (3.0) prepared by mixing 7 mL of triethylamine in 1 L purified water and the pH was adjusted to 3.0 ± 0.1 with concentrated orthophosphoric Acid. The solvent system was subjected to the process of sonication for 15 min to ensure proper mixing of the mobile phase. The mobile phase was filtered through a membrane filter with 0.45 µm pore size to remove any insoluble particulates.

2.5. HPLC Procedure

The RP-HPLC analysis was conducted using an Agilent 1220 LC system with a diode array detector (DAD). The injection volume was set to 20 µL. The elution run time of 15 min was employed in the isocratic mode of the mobile phase. The flow rate of 1 mL/min at 0-600 psi pressure was optimized with the column temperature maintained at 35 ^{o}C used for separation. The wavelength of the detection was set at 290 nm. A literature search was done and after the trials of various methods reported, the method from the Ministry of Health, Sultanate of Oman

was finally selected. In one of the studies retention times of the tadalafil was found to be 4.46 min when the solvent system selected was acetonitrile: acetate buffer pH 2.8 in the ratio of 55:45 v/v (Sutar *et al*., 2008).

2.6. Calculation

The following formula was used to find out the adulteration in the honey samples:

$$\left(\frac{mg}{g}\right) = PA * \frac{SC}{PA} * PS$$

PA: Peak area of sildenafil or tadalafil in sample
SC: Standard concentration
PS: Potency of standard

2.7. Validation

2.7.1. *Method development*

A single reversed-phase (RP)-HPLC method was used by applying in different compositions, ratios, and pH of mobile phases for the simultaneous estimation of sildenafil and tadalafil. This method shows a good linearity within the concentration range of 2-10 µg/mL for both analytes. The **s**ystem suitability tests were performed on freshly made 5 replicates from the standard stock solution of sildenafil and tadalafil. System suitability was evaluated using parameters such as retention time, % RSD of retention time, peak area, and theoretical plates.Validation of the developed RP-HPLC method was performed according to ICH guideline parameters and referencing some research papers (Agency, 1995; Pannu *et al*., 2022)**.**

3. FINDINGS

3.1. Validation

A rapid, sensitive, and robust RP-HPLC method was used for the simultaneous estimation of sildenafil and tadalafil in honey samples. This reported method is precise, accurate, linear, and specific to the parameters of validation. The linearity was found in the concentration range of 02-10 µg/mL for both analytes with a correlation coefficient of 0.9991 for sildenafil and 0.9995 for tadalafil.

This method was found to be sensitive to low LOD Values i.e., 0.42 µg/mL, and 0.30 µg/mL for sildenafil and tadalafil respectively. This method is validated at LOQ level i.e., 1.28 µg/mL and 0.90 µg/mL for sildenafil and tadalafil respectively. The % RSD for the method precision and robustness parameters were found within limits. The validation parameters are summarized in Table 2.

Table 2. Validation parameters.

Parameters	Analyte	
	Sildenafil	Tadalafil
Absorption maxima λ_{max} (nm)	290 nm	290 nm
Linearity (µg/mL)	2-10	2-10
Correlation coefficient(R^2)	0.9991	0.9995
Regression equation (y)	366.53x -30.143	788.53x + 60.19
Limit of detection (µg/mL)	0.42	0.30
Limit of quantification (µg/mL)	1.28	0.90
Intraday precision (n=6) (% RSD)	0.821	0.684
Interday precision (n=6) (% RSD)	0.877	0.432

3.2. HPLC Data

We validated a new, simple, highly sensitive RP-HPLC method for simultaneous estimation of sildenafil and tadalafil in marketed honey. The chromatographic separation was achieved on an ODS C18 Agilent (250 × 4.6 mm, 5µ) column with a mobile phase consisting of buffer: methanol: acetonitrile (5.8:2.5:1.7). Both peaks were resolved at a flow rate of 1.0 mL/min with 35 °C column temperature. The retention times of sildenafil and tadalafil were found to be 6 min and 11 min respectively. The method was found to be linear in the concentration range of 2-10 µg/mL for both analytes with correlation coefficients of 0.9991 and 0.9995 respectively. The method was successfully applied for detecting the sildenafil or tadalafil level in selected honey samples marketed in Oman.

3.2.1. *Assay of sildenafil and tadalafil in honey samples*

The results of the assay used for checking the content of sildenafil and tadalafil in marketed honey samples are shown in Table 3. The drug sildenafil or tadalafil were eluted at 6.244 and 10.898 minutes, respectively (C_{18} column Agilant coupled with DAD; mobile phase ratio buffer: methanol: acetonitrile in the ratio of (5.8:2.5:1.7) whereas in another study carried out by Abdelshakour *et al* obtained the retention time of sildenafil or tadalafil to be at 4.94 and 10.40 minutes (C_{18} column HPLC-UV; mobile phase with acetonitrile and 0.05% formic acid). The results showed that 4 honey samples were found to have sildenafil content. The contents of sildenafil were found to be higher in samples HAD5 and HAD1 and were 22.65 and 10.25 mg/g of the honey. The other two samples HAD6 and HAD7 showed much lower sildenafil contents i.e., 0.325 and 0.350 mg/g of honey tested. The study conducted in the Sultanate of Oman among 33 samples of herbal medicines and food supplements also showed that sildenafil was the most common adulterant with a percentage range from 0.7 to 12 wt % (Al Lawati *et al*., 2017). Similarly, sildenafil was considered as primary active compound detected in the range of 1.1 mg/sachet to 124 mg/sachet in a study carried out by Sirhan *et al*., in honey-mixed herbal sachets (Ala'Y *et al*., 2023). Out of 50 samples studied by Abdelshakour *et al*., almost all samples labeled to contain herbal or natural ingredients were found to be adulterated with PDE5 inhibitors such as sildenafil and tadalafil without being labeled in the package and sold as safe and natural products (Abdelshakour *et al*., 2021). Our study showed that 2 of the tested honey samples were adulterated with tadalafil. Among them, the sample HAD4 contains the maximum concentration of tadalafil i.e., 1.24 mg/g of tested honey. The HAD6 has a much lower concentration of tadalafil 0.7 mg/g of the honey sample tested, whereas Lawati *et al* reported tadalafil content in one of the food samples with 39 wt % (Al Lawati *et al*., 2017). In another study, Ala'Y *et al* detected tadalafil in few samples in the concentration range of 0.67 mg/sachet to 76.6 mg/sachet (Ala'Y *et al*., 2023). The HPLC chromatogram results are represented in the Figure 1.

Table 3. Assay determination of sildenafil and tadalafil in mg/g of honey sample by RP-HPLC.

Sample	Estimation (mg/g of sildenafil in honey samples)	Estimation (mg/g of tadalafil in honey samples)
HAD1	10.25	x
HAD2	x	x
HAD3	x	x
HAD4	x	1.248
HAD5	22.65	x
HAD6	0.325	0.7
HAD7	0.35	x

x: means no sildenafil/tadalafil present

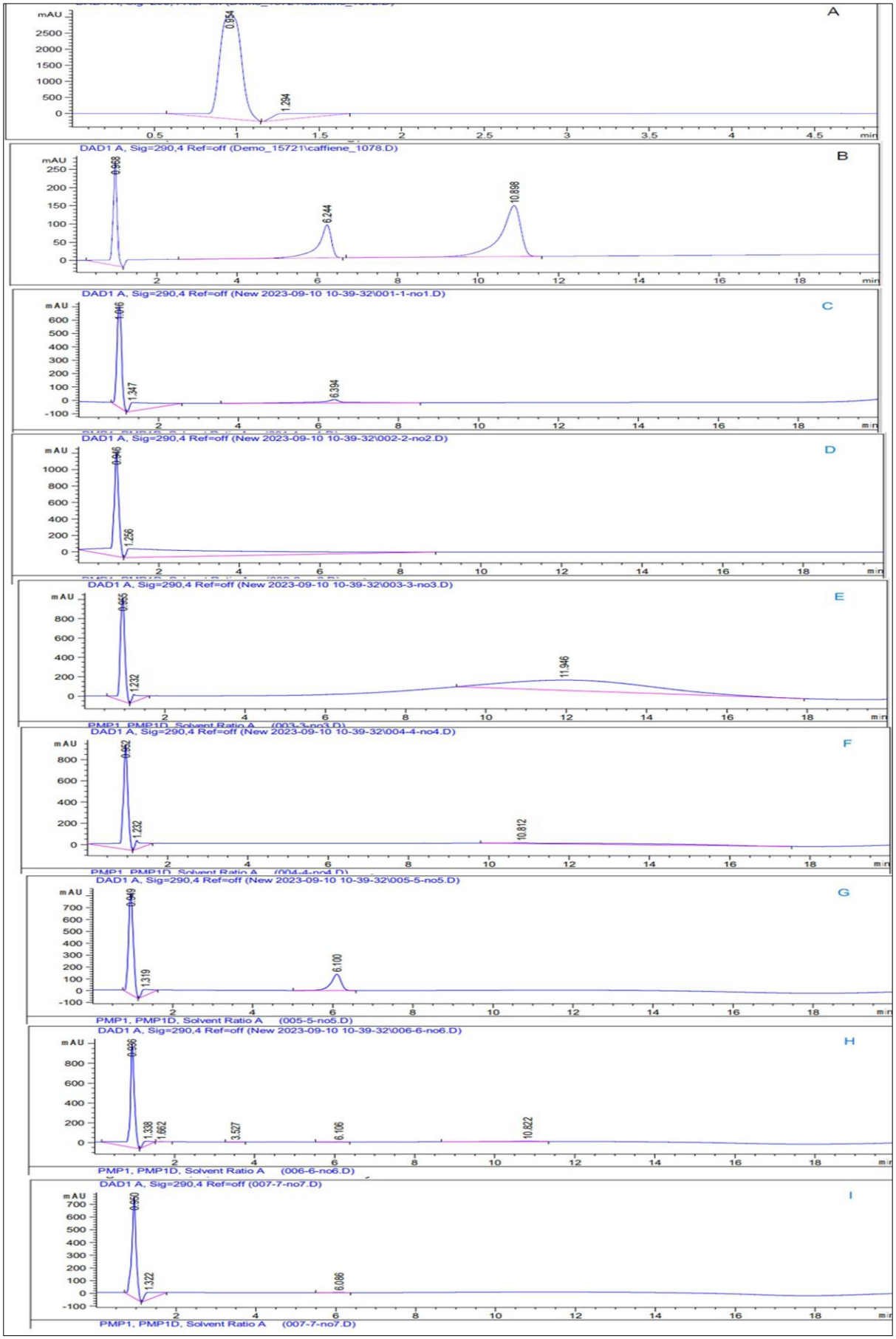

Figure 1. HPLC chromatogram of the solvent methanol (A), Combined sildenafil + tadalafil (B), HAD1 (C), HAD2 (D), HAD3 (E), HAD4 (F), HAD5 (G), HAD6 (H), HAD7(I) at a sample concentration of 10 mg/mL.

4. CONCLUSION

In this study, the HPLC procedure was applied for the determination of the sildenafil or tadalafil in the honey samples. HPLC is a valuable extension of the screening of the sildenafil or tadalafil not agreeable to UV-Vis as the drugs sildenafil and tadalafil have λ_{max} absorbance overlapping. A total of 7 honey samples were identified as the honey samples claimed to provide sexual enhancement and boost reproductive health. Among the tested samples 4 of the samples were found to be adulterated with sildenafil and 2 of the samples with tadalafil. The total sildenafil contents were found to be varied as samples HAD5 and HAD1 showed the maximum concentration whereas HAD6 and HAD7 had much lower concentrations of sildenafil. The tadalafil content was found to be maximum with sample HAD4 whereas HAD6 contains much lower contents of tadalafil. One of the samples HAD3 showed broad peaks at 11.946 which may be due to the presence of two or more poorly resolved compounds. These results provide the high quantitative capability of the HPLC method for determining honey adulteration with sildenafil or tadalafil. Furthermore, detailed studies must be carried out to confirm the findings of the current study, and novel analytical methods be developed to detect the level of other possible adulterants in this valuable product. Additionally, it would be interesting to perform other adulterant studies to determine the quality of honey samples in Oman and their impact on the health of consumers.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: EBS/PRES0401-COP/04/2022-23 College of Pharmacy, National University of Science and Technology, Azaiba, Muscat, Sultanate of Oman.

Authorship Contribution Statement

H.H. Aldhakhri: Investigation; Methodology. **F.H. Alqassabi**: Investigation; Methodology. **I.A. Alrasbi:** Investigation; Methodology. **D.A. Alhinai:** Investigation; Methodology. **SSR Al Salt:** Analyzed and Interpreted data **S. Pannu:** Formal analysis, supervision. **S. Al Balushi:** Formal analysis, supervision. **S.A. Khan:** Formal analysis, writing. **Md J. Akhtar:** Conceptualization, Data curation.

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Research Article

In-silico analysis of stress tolerance and secondary metabolite production in wild *Sesamum mulayanum* compared to cultivated *Sesamum indicum*

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Abstract: Sesame (*Sesamum indicum*) is a globally cultivated oilseed crop known for its nutraceutical and pharmaceutical significance. Its rich content of antioxidant lignans, mono- and polyunsaturated fatty acids, vitamins, minerals, carbohydrates, and proteins contributes to its importance. To enhance understanding of sesame's genetic potential for crop improvement and utilization, transcriptome data from two sesame species, *Sesamum indicum* and *Sesamum mulayanum*, at two developmental stages (10 and 30 days after pollination, DAP) were analyzed using the Galaxy platform to identify differentially expressed genes. The results showed that 170 genes were up-regulated, and 46 genes were down-regulated. Gene ontology analysis revealed that up-regulated genes were involved in diverse molecular functions and biological processes related to defense response to nematode, systemic acquired resistance, abscisic acid response, and detoxification, among others. Similarly, pathway analysis revealed that the up-regulated genes were involved in pathways related to plant defense, secondary metabolite synthesis, fatty acid synthesis, and phenylalanine, tyrosine and tryptophan biosynthesis. A network analysis was also predicted for describing the interaction of secondary metabolites and stress tolerance genes. The results of the present study provide new insights into the genetic and genomic understanding of sesame, thereby helping in future crop improvement.

1. INTRODUCTION

Sesame (*Sesamum indicum*) is cultivated in different parts of the world. Countries like Myanmar, India, and China are the bulk producers of sesame. Sesame seeds are rich in antioxidant lignans mono and polyunsaturated fatty acids which make it nutritionally and pharmaceutically important oilseed crop (Dar *et al*., 2019). The oil content of sesame varies from 40% to 60%. When compared to the world's average production of sesame, productivity is low in India(Venkataravanappa, 2017). *Sesamum indicum* (Figure 1a) is a cultivated species of the genus Sesame. Sesame has nearly 26 species revised and updated by Nimmakayala *et al*., (2011) which serves as a rich source of biodiversity. Sesame seeds have a highly roasted flavor suitable for making cakes, flour, oil, and paste (Yaseen *et al*., 2021). The seed color of sesame ranges from yellow, grey, brown, black, white, red, and tan. Oil content in white seeds is greater than in other dark color seeds (Yaseen *et al*., 2021). In contrast, black color sesame seeds with

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a slightly lower oil content have a higher content of sesamin, sesamolin, total lignans, and oleic acid (Dar *et al*., 2019).

In addition to the above, sesame oil also contains vitamin E, minerals, such as copper, calcium, iron, zinc, phosphorous, carbohydrate, and proteins rich in methionine (Yaseen *et al*., 2021). Different parts of sesame have been used to treat various conditions like asthma, wound healing, ulcers, inflammations, hemorrhoids, and amenorrhea (Mili *et al*., 2021). One of the varieties of sesame Milyang 74 (M74) extract has high lignan content (17.71 mg/g) which has beneficial effects in the treatment of Alzheimer's disease (Kim *et al*., 2023). Various phytochemical compounds have been identified, isolated, and characterized from seeds, plant organs, and seed oil. Phytochemicals include polyphenols, phytosterols, lignans, phenols, naphthoquinones, anthraquinones, cerebroside, triterpenes, sugars, and other organic compounds. These components are present equally or in higher amounts in the wild varieties of sesame (Mili *et al*., 2021). Comparative genomics and transcriptomic data of wild tetraploid sesame, (*S. schinizianum*) with *S. indicum* have provided insights into lignan synthesis pathways (Wang *et al*., 2023). Indian subcontinent is rich in sesame biodiversity hence identification and exploitation of the wild relatives of sesame to improve the ruling varieties are the need of the hour.

Cultivated sesame is susceptible to most of the biotic and abiotic stresses like cold, drought, phyllody, wilt, fungal diseases, etc., (Dutta *et al*., 2020). Important reasons for the low productivity of sesame include a low harvest index, a lack of high-yielding varieties having resistance to biotic and abiotic stresses. Phyllody is the abnormal development of floral parts into a leafy structure. It is generally caused by phytoplasma or viral infections, though it may also be because of environmental factors that result in an imbalance in plant hormones. The wild species *S. mulayanum* (Figure 1b) from the same genus shows resistance to many pathogens (Dutta *et al*., 2020). It can serve as a gene pool for transferring resistant traits since interspecies crosses between these two species are possible without pre-fertilization barriers (Kulkarni *et al*., 2017). *S. mulayanum* is found to be resistant to phytoplasma with a mean incidence level of less than 5% of phyllody when artificially infected. Hybridization and back-crossing studies have revealed that phyllody resistance is governed by a single recessive and dominant gene in cultivated and wild species respectively in sesame (Singh *et al*., 2007). Many diseases are spread by insect vectors, hence insect resistance can provide increased disease resistance. Daphedar *et al*., 2024 reported that phenylalanine is one of the important secondary metabolite from the phenyl propanoidpathway which helps plants to resist various microorganisms including pathogens, and withstand biotic and abiotic stresses.

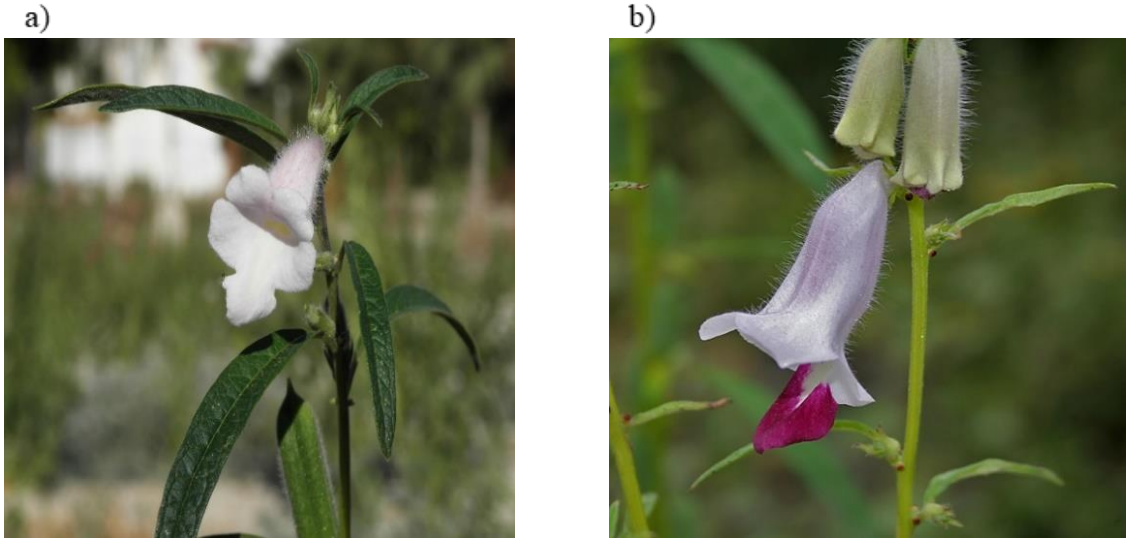

Figure 1. Comparison of plant architecture and flower color of *S. indicum* and *S. Mulayanum.*

Understanding molecular mechanisms of biotic and abiotic tolerance of sesame help not only to find the genes related to these traits but also the associated phenotypic traits that help in selection and follow speed molecular breeding approaches. Towards this end, an exclusive sesame database called Sinbase-2.0 comprising sequences, linkage maps, transcriptome, proteome, QTL, Variants, functional markers, and genes were developed (Wang *et al*., 2021). Earlier, Sesame FG was a database to get all genotypic and phenotypic data on sesame was available (Wei *et al*., 2017). Dossa *et al*., (2017) reported that the sesame crop has moved from an orphan status to a genomics resource-rich crop. This will enable the scientific community to explore the potential of the crop by integrating genomic, transcriptomic, and metabalomic data. Accordingly, to Zhang *et al*., 2021) have integrated miRNA, transcriptome, and degradome data to understand the lipid and fatty acid synthesis during seed development. In this paper, we try to integrate transcriptome data of the cultivated and wild species of sesame to to understand the genetic potential of these species for crop improvement and exploitation purposes.

2. MATERIAL and METHODS

2.1. Data Extraction

Dutta *et al*., 2022 carried out transcriptome analysis of developed seeds from two sesame types named *Sesamum indicum* (from NBPGR germplasm: IC131989) and wild *Sesamum mulayanum* (a generous gift from Mr. K Masuda, Department of Biology, Faculty of Science, University of Toyama, Japan). RNA sequencing analysis was carried out for two genotypes at 10 and 30 DAP and SRA (sequence read archives) data were submitted to the NCBI bio project (Accession: PRJNA644139). For this study, SRA data submitted by Dutta *et al*., 2022 were used. For *Sesamum indicum* 10DAP: Run- SRR12153209 & 30DAP: Run-SRR12153208 and for *Sesamum mulayanum* 10DAP: Run-SRR12153201 & 30DAP: Run- SRR12153200 were used to identify the differentially expressed genes in sesame to explore the genetic potential of these crops for crop improvement.

2.2. Identification of Differentially Expressed Genes (DEG)

To process the RNA sequences and to identify the differentially expressed genes, tools and software present in the Galaxy platform were found suitable. A flowchart adopted for RNA analysis using the Galaxy platform for the DEG identification is given in Figure 2. SRA data downloaded from NCBI was uploaded to the tool and different tools were used to find the differentially expressed genes. The quality of the SRA data was checked by using FastQC (version 0.11.8). Trimmomatic (version 0.38) was used to remove the adapters and low-quality reads in the SRA data. The reads were mapped to their reference genome by using the tool HISTAT2 (version 2.1.0).

The reference genome of *S. indicum* was used because the *S. mulayanum* genome was not yet annotated. A BAM file was generated as an output of HISAT2 which contains the aligned reads. Then the BAM file was loaded to Stringtie (version 2.1.1) which quantified the aligned reads to the reference genome. To generate non-redundancies set of transcripts in all the above RNA samples Stringtie merge was performed. As a result, Stringtie files were merged as a single file. Deseq2 (version 1.22.1) was used to estimate the variance-mean dependence in count data generated by stringtie (using a stringtie merge file). The output DEseq2 file was loaded to the tool- Annotate DESeq2/DEXSeq output tables (version 1.1.0). The resultant table file contains gene identifiers with P-value and log (FC) normalized fold change values. Results of the Galaxy contain only the gene identifiers of the differentially expressed genes. To convert the gene identifiers to gene names KEGG Mapper was used where *S. indicum* was used as an organism code.

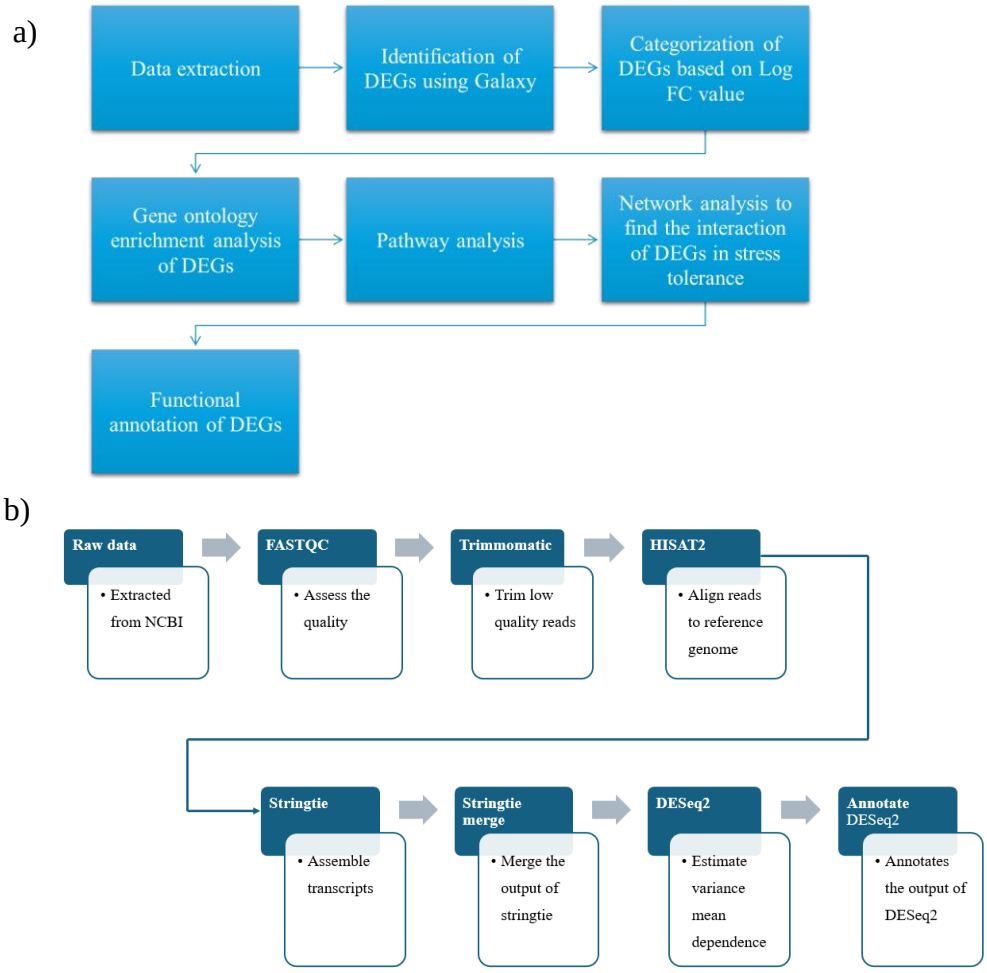

Figure 2. a) Flow chart of steps followed b) Pipeline used to find the DEGs of *S. mulayanum*.

2.3. Categorization of Degs

Based on P-value and Log FC, every gene found using KEGG Mapper was further classified. P-values less than 0.05 indicate statistical significance for a gene and based on the log Fold Change (FC) values, the gene was categorized as upregulated or downregulated. Genes having log (FC) < 0 was categorized as downregulated and log (FC) > 0 as upregulated Then, the upregulated genes were categorized into four classes into which the elevated genes were subsequently divided based on molecular functions and biological processes which are described in section 3.2.

2.4. Gene Ontology (GO) Enrichment Analysis

Gene Ontology (GO) provides an idea about the molecular function, important biological processes and potential applications of genes. Gene Ontology analysis has different categories that includes Molecular function (MF), cellular component (CC), and biological processes (BP) (Li *et al*., 2019). To understand the functions and proccesses of differentially expressed genes, GO enrichment analysis was performed by using Quick go tool (https://www.ebi.ac.uk/QuickGO/).

2.5. Pathway Analysis

Pathway analysis is the process of classifying large gene sets by the KEGG database ((Li *et al*., 2019). For doing pathway analysis different tools were referred that includes PANTHER (http://www.pantherdb.org/pathway/) and KEGG pathway database. Finally, Pathway analysis of upregulated genes was done by using the KEGG pathway database (https://www.genome.jp/kegg/pathway.html). Gene symbols were uploaded to the tool and sind (*S. indicum*) was used as an organism code.

2.6. Network Analysis of Secondary Metabolite and Stress-Related Genes

The DEGs which significantly upregulated for secondary metabolite production and stress response which includes biotic and abiotic stresses in *Sesamum mulayanum* were analyzed using (https://string-db.org/) STRING (Version -11.5) for their inter relatedness. As expected, it showed significant interconnection between the genes. Since the genome of *S. mulayanum* was not annotated yet, *Arabidopsis thaliana* was used as a reference organism.

2.7. Functional Annotation of The DEGs

The DEGs were searched and correlated with published literature to find exactly the type of stress tolerance the upregulated genes are involved in. The results of the upregulated genes involved in various types of stress tolerance mechanisms including biotic and abiotic stresses. Among the various biotic stresses, phyllody is the major disease that extremely affects the sesame growth and yield. Phyllody resistance can also be gained by insect tolerance/resistance.

3. FINDINGS

3.1. Up-Regulated *S. mulayanum* Genes During Seed Development

The hierarchical clustering heatmap shows that samples at the same developmental stages are clustered together and shown in Figure 3a. The PC1 variance is about 82% in the principal component analysis plot which means the samples have high variance in gene expression and the samples at 30 DAPs are highly separated than the samples at 10 DAPs shown in Figure 3b.

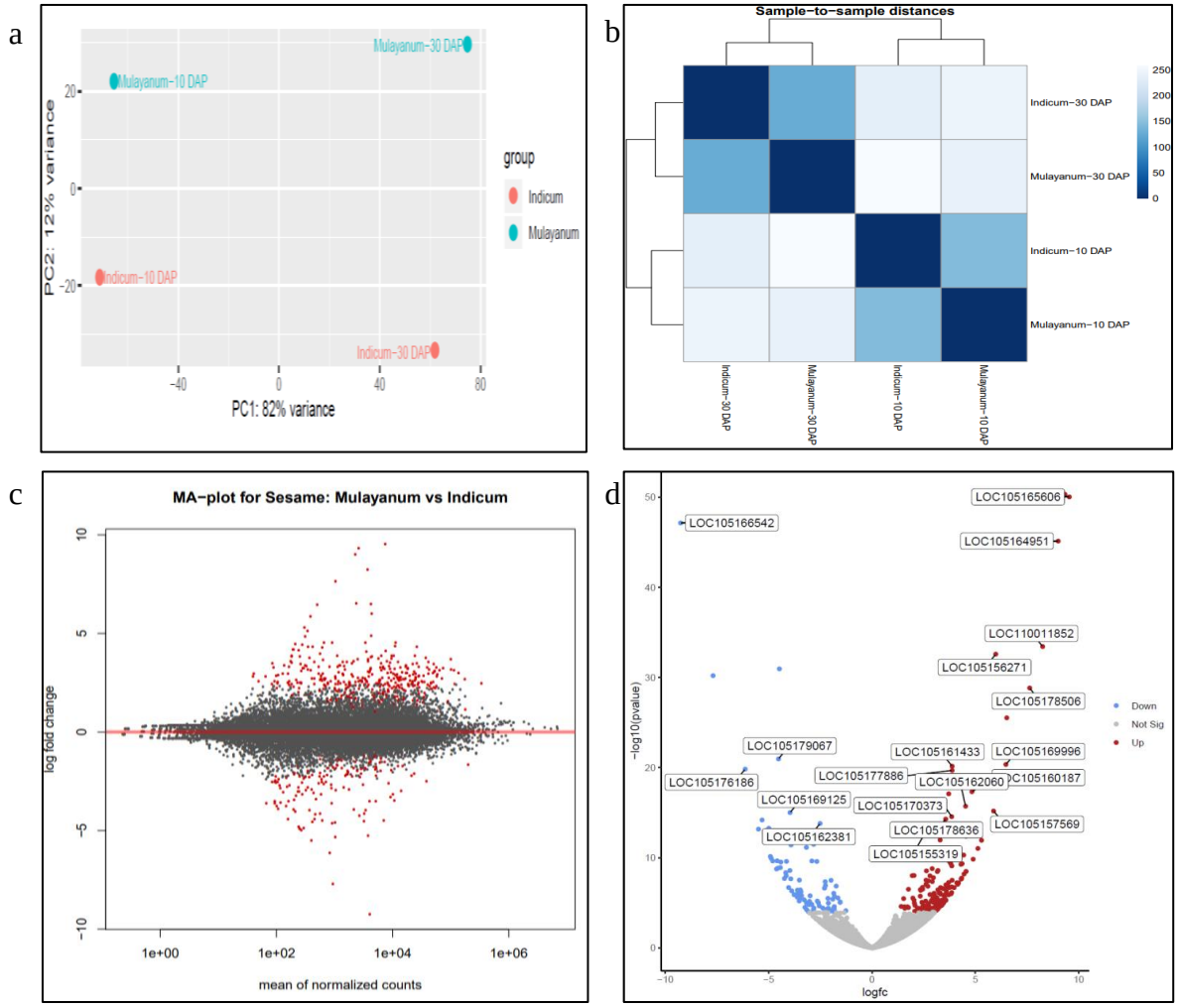

Figure 3. Visualization of DEG analysis: a) PCA plot using the log2 transformed counts b) HC heatmap of sesame sample (at 10 DAP and 30 DAP) c) MA plot representing the normalized fold change with the mean of normalized counts d) Volcano plot representing the top significant genes.

This indicates that the gene expression variation is high at 30 DAPs. In the Mean average difference plot, the red dots represent the significant genes, it shows that most of the significant genes are up regulated ones shown in Figure 3c. A volcano plot was used to highlight the top significant genes which is shown in Figure 3d. 216 genes were found to be significantly differentially regulated. Out of this,170 genes were up regulated, and 46 genes were down regulated.

The top significant genes include F-box/kelch-repeat protein At3g23880-like, MYB-like transcription factor ETC1, cytochrome P450 94C1, exocyst complex component EXO70A1-like, probable calcium-binding protein CML1, peptidyl-prolyl cis-trans isomerase FKBP15-3-like, ethylene-responsive transcription factor TINY, exocyst complex component EXO70A1, salicylate carboxy methyltransferase, 4,5-DOPA dioxygenase extradiol, 3-ketoacyl-CoA synthase 11, anthocyanidin 3-O-glucosyltransferase 2, elongation factor 1-beta-like, putative disease resistance protein RGA1, carotenoid 9,10(9',10')-cleavage dioxygenase 1-like and dicer-like protein 4 isoform X3.

The upregulated genes were then separated into 4 different categories. They are represented in the pie chart shown in Figure 4. They were categorized as annotated protein coding genes, transcription factors, putative genes (proteins having similar function) and uncharacterized genes.

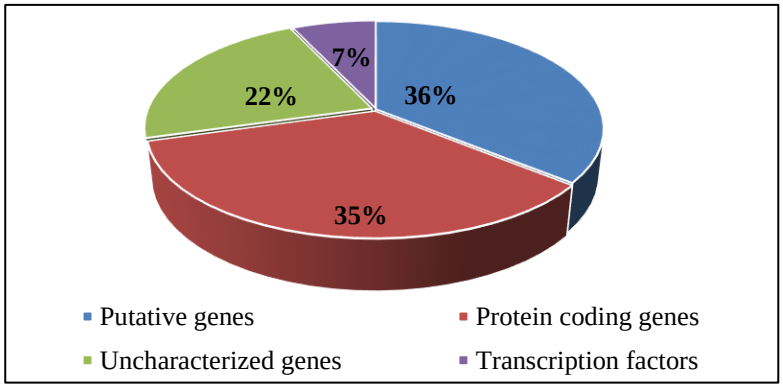

Figure 4. Categories of Differentially expressed genes (DEGs) in *S. mulayanum* Vs *S. indicum.*

3.2. Augmentation of Stress-Responsive Genes in *S. mulayanum*

Gene ontology analysis showed that the DEGs were involved in various molecular functions as well as diverse biological processes. The upregulated genes(170) were represented by 36 different molecular functions and 38 different biological processes and for the (46) downregulated genes, 14 different molecular functions and 15 different biological processes were involved. The Predominant biological functions of upregulated genes related to resistance are defense response to nematode (GO:0002215), systemic acquired resistance and salicylic acid-mediated signaling pathway (GO:0009862), response to abscisic acid (GO:0009737), detoxification of zinc ion (GO:0010312), salicylic acid biosynthetic process (GO:0009697), cellular oxidant detoxification (GO:0098869), response to ethylene (GO:0009723). The important biological processes of differentially expressed genes related to resistance and the corresponding expression level of the genes were represented by the bar graph shown in Figure 5.

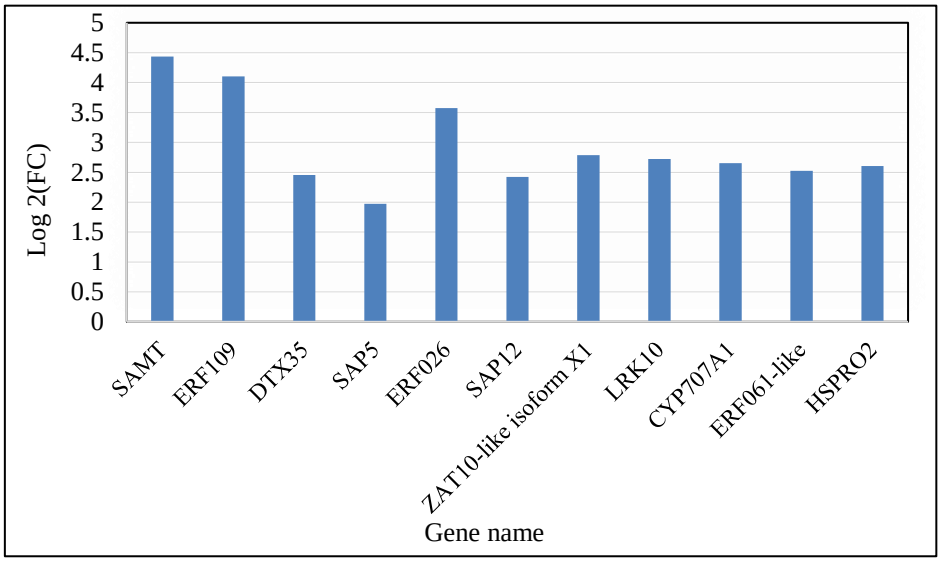

Figure 5. Stress-related *S. mulayanum* genes and their fold changes with reference *S. indicum* expression levels.

As a result, of comparative analyses, several upregulated genes were found to be involved in various biotic and abiotic stresses in different plant species. There are 23 biotic stress tolerance genes, and 26 abiotic stress tolerance genes were found. Upregulated genes involved in biotic stress tolerance were identified through literature analyses are shown with its log (FC) value in Table 1. Similarly, genes related phyllody disease resistance were specifically analyzed.

Table 1. Upregulated genes of *S. mulayanum* in biotic stress responses along with its Log2(FC) value.

Genes	Log2(FC) value
Probable calcium-binding protein CML18	3.88
Anthocyanidin 3-O-glucosyltransferase 2	3.57
Salicylate carboxymethyl transferase	4.44
4,5-DOPA dioxygenase extradiol	2.91
Polyubiquitin	1.95
3-ketoacyl-CoA synthase 11	3.71
Probable calcium-binding protein CML18	3.58
Mitogen-activated protein kinase kinase kinase NPK1	3.86
Syntaxin-121	3.31
Glutathione S-transferase F12	3.82
Probable protein phosphatase 2C 63	3.14
Putative calcium-binding protein CML23	3.34
Calmodulin-binding protein 60 C isoform X2	1.79
Calcium-dependent protein kinase 11 isoform X1	2.38
Zinc finger AN1 domain-containing stress-associated protein 12	2.42
F-box/kelch-repeat protein At3g23880-like	9.55
Elongation factor 1-beta-like	2.50
Calcium-binding allergen Ole e 8-like	2.99
Ethylene-responsive transcription factor ERF109	4.10
Nematode resistance protein-like HSPRO2	2.61
Glutathione S-transferase F12	3.82
Peroxidase 25-like	2.72
Linoleate 13S-lipoxygenase 3-1, chloroplast	2.71

Biosynthetic process and other includes L-phenylalanine biosynthetic process (GO:0009094), thiamine biosynthetic process (GO:0009228), chorismate biosynthetic process (GO:0009423), glutathione biosynthetic process (GO:0006750), coenzyme A biosynthetic process (GO:0015937), protein targeting to ER (GO:0045047), CAAX-box protein maturation (GO:0080120), intermembrane lipid transfer (GO:0120009), ethylene-activated signaling pathway (GO:0009873), regulation of exocyst localization (GO:0060178), cell wall modification (GO:0042545), mitochondrial calcium ion transmembrane transport (GO:0006851), xyloglucan metabolic process (GO:0010411).

Biological processes which are highly enriched by downregulated genes include fatty acid beta-oxidation (GO:0006635), ethylene mediated signaling pathway (GO:0009866), carotenoid biosynthetic process (GO:0016117), translational termination (GO:0006415), induced systemic resistance (GO:0009682), phosphatidylinositol dephosphorylation (GO:0046856), trans sulfuration (GO:0019346), induced systemic resistance and jasmonic acid mediated signaling pathway (GO:0009864).

3.3. Secondary Metabolite and Stress Tolerance Pathways are Linked

Pathway analysis of the upregulated genes indicated that the genes are involved in various pathways like Shikimate pathway, Betalain biosynthesis, Terpene biosynthesis, Carotenoid biosynthesis, MAPK signaling pathway, SNARE interactions in vesicular transport, Inositol phosphate metabolism, Fructose and mannose metabolism, pentose phosphate pathway and Monolignol biosynthesis. The secondary metabolite-producing gene's pathway and its applications are given in Table 2. Some of the genes of *S. mulayanum* involved in different pathways which directly (plant pathogen interaction, secondary metabolites, and pest resistance) and indirectly (synthesis of amino acids, phenylalanine, tyrosine synthesis and fatty acid synthesis) involved in plant defense against biotic and abiotic stresses shown in Figure 6.

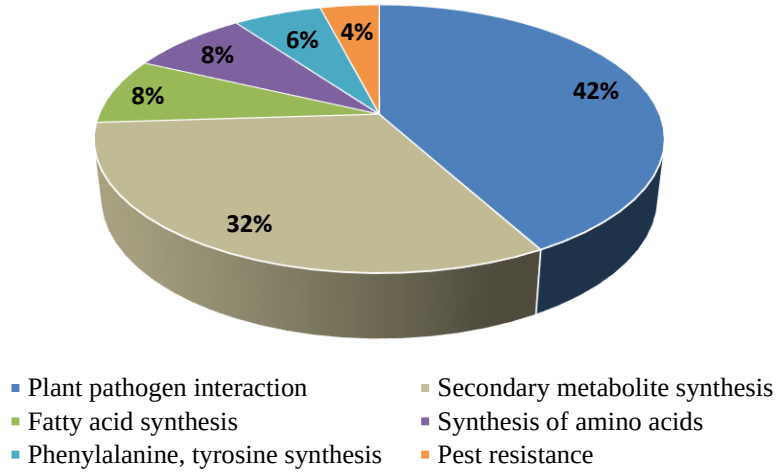

Figure 6. Upregulated genes of *S. mulayanum* involved in pathways connected to plant defense.

A network analysis was predicted for relating the interaction of secondary metabolites and stress tolerance genes. The network is generated between the secondary metabolites producing genes (PAL, LDOX, CM1, CYP707A1) with biotic (UFGT, KCS1, GSTF12, At4g33920) and abiotic stress tolerance genes (PUMP5, RDUF2, CCD4, GOLS1) are shown in Figure 7. Genome of *S. mulayanum* has not been annotated yet. Hence, *Arabidopsis thaliana* was used as a reference. The network generated was found to have 12 nodes and 10 edges with an average node degree of about 1.67. PPI enrichment P value is 2.53e-11. This shows that the network has significantly more interactions than expected. Totally, there are 16 secondary metabolite producing genes were found by correlating the literatures. Out of 16 genes, only a few formed the network with the biotic and abiotic genes. This is because the reference organism (*Arabidopsis thaliana*) does not possess certain genes that were present in *S. mulayanum.*

Table 2. Major secondary metabolite-producing genes of *S. mulayanum* and their application.

Genes involved in secondary metabolite synthesis	Products of the pathway	Applications
Probable carotenoid cleavage dioxygenase 4	Carotenoids - isoprenoids, terpenoids or terpenes	Antioxidants, light-harvesting pigments, and attractants for pollinators and seed dispersers. Response of plants to environmental stresses. Act in defense mechanisms (phytoalexins)
S-adenosylmethionine synthase 3	Ethylene	Plant growth regulation
Chorismate mutase 1, chloroplastic-like	Chorismate, shikimate	The synthesis of aromatic amino acids, p-aminobenzoic acid, folate
4,5-DOPA dioxygenase extradiol	Betalamic acid	Strong antioxidant activity, anticancer, hypolipidemic, hepatoprotective, anti-inflammatory, and antidiabetic activities
Geranylgeranyl pyrophosphate synthase, chloroplastic	Terpenoids	Signal molecules to attract the insects of pollination
	Polyketides	Anti-viral, anticancer, antifungal, and anti-microbial agents and neuroprotective
	Geranyl diphosphate	Gibberellin biosynthesis
Type I inositol polyphosphate 5-phosphatase 2 isoform X1	Phytic acid	The main storage form of phosphorus in the seeds
Fructokinase-2	D fructose 6 phosphate	Precursor of mucopolysaccharides (polysaccharides with nitrogen-containing components)
UDP-glucuronate 4-epimerase 1	Ascorbate	Antioxidant protects plants against oxidative damage
Phenylalanine ammonia-lyase	Monolignol	Biosynthesis of both lignans and lignin, regulating plant development, pigmentation, and UV protection
	Flavanone	Controlling plant development through their action in cell wall synthesis, and in defense against fungal pathogens.
Leucoanthocyanidin dioxygenase-like	Flavonoids	Important plant pigments for flower coloration, UV filtration, symbiotic nitrogen fixation,
Trans-cinnamate 4-monooxygenase-like	Quinone	Anti-proliferation and anti-metastasis effects
	Stilbenoid	Phytoalexins, which are antimicrobial compounds produced de novo in plants to protect against fungal infection and toxins
	Diarylheptanoid	A small class of plant secondary metabolites
	Gingerol	Powerful anti-inflammatory and antioxidant effects
Shikimate O-hydroxy cinnamoyl transferase-like	Cutin and suberin	Cell-wall associated glycerol lipid polymers,
	16 feruloyloxi palmitic acid	Antioxidants
Abscisic acid 8'-hydroxylase 1-like	Beta carotene	Protects plant cells against the destructive effects of ultraviolet light
	Abscisic acid	Role in various physiological processes of plants, such as stomatal closure, cuticular wax accumulation, leaf senescence, bud dormancy, seed germination, osmotic regulation etc..,
Linoleate 13S-lipoxygenase 3-1, chloroplastic	Oxo ode (octa decadienoic acid)	PPAR α agonist to decrease triglyceride accumulation in mouse primary hepatocytes
Peroxidase 25-like	Phenylpropanoid	Precursors for a wide range of secondary metabolites
Allene oxide cyclase, chloroplastic-like	Jasmonic acid	Endogenous growth-regulating substance

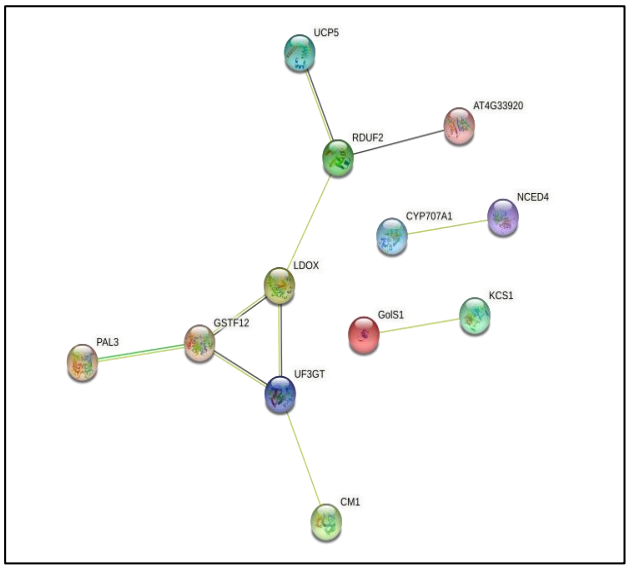

Figure 7. Interaction between secondary metabolites and stress tolerance (biotic and abiotic) genes of *S. mulayanum.*

4. DISCUSSION and CONCLUSION

4.1 Discussion

Transcriptome profiling is a comprehensive approach to understand the disease resistance pathways in sesame. In rice, sheath blight disease is studied by comparing the transcriptome profiles of resistant (CR1014) and susceptible (Swarna-sub-1) varieties (Bal *et al*., 2022). The molecular response, genetic basis and candidate genes of sheath blight was identified from differentially expressed genes. Molecular mechanism of phyllody resistance in sesame is poorly understood. Singh *et al*., (2007) have earlier reported the involvement of two independent non-allelic genes in phyllody resistance. In this study, we have compared the transcriptomic profiles of S. *indicum*, a phyllody susceptible, and *S. mulayanum*, a tolerant species to identify differentially expressed genes that are related with disease resistance. Transcriptome analyses of charcoal rot challenged *S. mulayanum* plants were used to identify defense related genes and pathways involved during plant- pathogen interaction (Dutta *et al*., 2020). In this study, among the DEGs, eleven S. *mulayanum* upregulated genes which are related to defense or stress tolerance are observed to have 2-4.5-fold increased expression than S. *indicum* and shown in Figure 5. A similar study in pepper root knot nematode had broadened the single loci concept to several interrelated pathways in transcriptome profiling (Hu *et al*., 2020). The gene responsible for exhibiting the systemic acquired resistance was up regulated and the gene responsible for induced systemic resistance was down regulated because the SRA data of *S. mulayanum* was obtained during seed development. This gene may be upregulated and shows its resistance whenever the plant is challenged with the pathogen. Induced systemic resistance (ISR) can be induced during any interactions between the plant and pathogens. Once it is activated, it elicits a set of localized responses in and around the infected host cells (Heil & Bostock, 2002). In the current transcriptome analyses S. *mulayanum* has revealed 42% of pathogenesis related genes which are up regulated than *S. indicum* are shown in Figure 6. This suggests that these genes and their pathways might contribute to the disease tolerance nature of *S. mulayanum.*

Plant secondary metabolites confer several functions to the plants. They improve the innate immunity of plants besides contributing to the growth, development, and nutritive value of the plant. The disease tolerance is recently proven by transgenic rice overproducing flavonoids exhibiting bacterial leaf blight resistance (Jan *et al*., 2021). Similarly, in sesame, greater accumulation of secondary metabolites is correlated with increased charcoal rot disease resistance (Chowdury *et al*., 2021). Hoda *et al*., 2024 reported that sesame seeds irradiated with

low doses of gamma rays showed an increased production and activity of peroxidase and polyphenol oxidase, phenolic, and lignan contents after germination when compared to non-irradiated sesame seeds. The increased production of secondary metabolites increased the resistance for charcoal rot disease caused by *Macrophomina phaseolina*. In this investigation, S. *mulayanum*, a disease tolerant wild species shows 32% of the upregulated genes to be involved in secondary metabolite production compared to the susceptible species which is shown in Figure 6. Genes like Peroxidase 25-like and phenylalanine ammonia lyase significantly upregulated in *Sesamum mulayanum* involved in the production of phenylpropanoid and monolignols which are the precursors for lignan, and phenolic compounds shown in Table 2. These increased secondary metabolites production made *Sesamum mulayanum* a natural tolerant species to various biotic stresses. Carotenoids, Betalamic acid, Ascorbate, Gingerol, and 16 feruloyloxi palmitic acid have strong antioxidant property. Antioxidants are present in higher amounts in the wild sesame species compared to cultivated gene pool (Pathak *et al*., 2020). Under stress conditions whether it may be biotic or abiotic, the production of reactive oxygen species (ROS) was increased in plants. This in turn induces oxidative stress. The increased oxidative stress, plants increase the production of lower molecular and higher molecular antioxidants. These in turn help to reduce the oxidative stress in the plant (Kasote *et al*., 2015).

The key genes related to environment stress in *S. mulayanum* are salicylate carboxy methyltransferase, ethylene-responsive transcription factor ERF025, protein DETOXIFICATION 35, zinc finger A20 and AN1 domain-containing stress-associated protein 5, rust resistance kinase Lr10-like and abscisic acid 8'-hydroxylase 1-like. Among those key genes, salicylate carboxy methyltransferase exhibits systemic acquired resistance and salicylic acid-mediated signaling pathway. Salicylic Acid (SA) plays an important role in both systemic and local defense responses in plants. Systemic acquired resistance (SAR) is a salicyclic acid (SA) dependent response that elicits long distance signaling mechanism. As a result, it provides broad spectrum and long-lasting resistance to secondary infections of the entire plant (Gao *et al*., 2015). Of the up regulated genes, salicylate carboxy methyl transferase was found to be the topmost gene (4.4-fold up regulated in *S. mulayanum* as compared to the *S. indicum*). This suggests the plausible role of such secondary metabolite genes in conferring disease resistance to *S. mulayanum*.

In our study, an exhaustive literature analysis was performed and a total of 16 secondary metabolite producing genes, 23 biotic stress tolerance genes and 26 abiotic stress tolerance genes were further analyzed for interaction. Out of this, four secondary metabolite producing genes (PAL, LDOX, CM1, CYP707A1) were found to interact with biotic and abiotic genes to form a significant network. Phenyl alanine ammonia lyase (PAL) is one of the major key enzymes which are involved in the phenyl-propanoid pathway. Major products of the pathway and product's applications are mentioned in Table 2. Major products are lignans and flavonoids which are involved in resistance against charcoal rot disease (Hoda *et al*., 2024). LDOX, one of the secondary metabolites producing genes which formed significant interaction with stress tolerance genes was involved in anthocyanin biosynthesis. Upon cold stress this gene was found to be expressed more in purple black carrot and helps the plant to withstand the stress (Dar *et al*., 2022). CYP707A1 is the loci where histone deacetylase HDA9 will bind and induce drought resistance of the plant. This mechanism was studied in Cai *et al*., 2022. Drought induced long intergenic noncoding RNA DANA1 interacts with DANA1-INTERACTING PROTEIN 1 (DIP1) which further increases the binding of HDA9 to CYP707A1 loci and increases the drought resistance of the plant. Few genes were removed and the reasons behind this could be that the novel secondary metabolite producing genes from *S. mulayanum* that were identified in this study were not included in the *A. thaliana* PPI network in STRING previously.

The secondary metabolites involved in plant growth and development are induced by compounds like ethylene, and gibberellic acid (GA) etc., GA plays an important role in seed

germination, internode elongation, flower initiation, development, and abscisic acid (ABA) is involved in maintenance of dormancy. Gibberellins (GA) plays a vital role in embryo development. GA is one of the important constituents that regulates temporal organization of maturation phase (Gupta & Chakrabarty, 2013). Secondary metabolites accumulation during seed development in sesame species is correlated to their transcriptomic profile. In *S. radiatum*, another wild species, novel secondary metabolites, jan sesangolin and episesantalin accumulation was shown to be correlated with their corresponding transcript levels at 35 DAPs (Harada *et al*., 2020).

In another study, secondary metabolites of wild sesame, *S. angustifolia* a potential vegetable crop, is found to have higher nutritive value that satisfies daily nutritional requirements of vitamins and micronutrients (Maina *et al*., 2019). Similarly, the dried leaves of *S. radiatum* have been shown to possess significant amounts of micro and macro nutrients, proteins, phenolic compounds, and antioxidants (Catarino *et al*., 2019). Sesame wild species, *S. lanciniatum, S. radiatum,* and *S. indicum* subsp. *malabaricum* have higher phenolic contents and antioxidants (Pathak *et al*., 2020). Accordingly, *S. mulayanum* also might also have several secondary metabolites correlating to their gene expression during seed development. Correspondingly, sesamin and total lignan content in *S. mulayanum* were found to be higher than the other cultivated Indian varieties (Pathak *et al*., 2015). The above observations suggest that *S. mulayanum* could be a potential oilseed crop that can be exploited for its valuable oil, secondary metabolites, and disease tolerance nature.

4.2 Conclusion

The farmed *S.indicum* contains a significant number of secondary metabolites in addition to its high oil content. However, a number of diseases, including phyllody, leaf spot, powdery mildew, and root and stem rot, can affect planted sesame and severely reducing its oil yield productivity. Improved disease tolerance commercial varieties urgently need to be substituted. In order to achieve this, this study assessed and analyzed a wild sesame *S.mulayanum*'s genetic potential for the synthesis of lignans and other secondary metabolites. It was found that nearly 74% of the upregulated genes in *S.mulayanum* are either involved in plant pathogen interaction or secondary metabolite production which interact with stress tolerance genes. The secondary metabolites of *S. mulayanum* can be directly used for nutraceutical or pharmaceutical purposes and the genetic potential of the crop can be exploited in breeding programs to improve the domesticated indicum varieties.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Selvi Subramanian: Supervision, validation of results, original draft revision, resources. **Dharanidharan Manivannan**: Investigation, methodology, visualization, software, formal analysis, and writing -original draft.

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Research Article

Cytotoxic and apoptotic effect of *Lemna minor* L. extract on human osteosarcoma (Saos-2)

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Abstract: Osteosarcoma is a malignant form of bone cancer that responds poorly to chemotherapy and has a significant incidence of recurrence. Recent studies suggest the need for new natural agents to support treatment. Lemna minor is a macrophyte frequently used in traditional treatments and is known to have high antioxidant and antimicrobial properties due to the phenolic compounds it contains. This study investigated the anticancer activity of the *L. minor* on Saos-2 cancer cells and the apoptosis pathways. *L. minor* was extracted in ethanol by microwave technique. The extract showed cytotoxic activity (59-79%) on Saos-2 cells but did not harm healthy human bone cells (hFOB). Expression of bax, bcl-2, caspase-3, and caspase-8 genes was investigated by RT-PCR to examine the apoptosis-inducing pathways. RT-PCR analysis revealed that L. minor induced apoptosis via a mitochondria-dependent pathway by affecting the bax/bcl-2 ratio. The study suggests *L. minor* as a promising natural agent for bone cancer treatment.

1. INTRODUCTION

Osteosarcoma is a malignant bone tumor that mainly affects children, young adults aged 0-24 years, and those over the age of 60 (Beird *et al.*, 2022; Janani *et al.*, 2022). Advances in treatment have led to a rise in survival rates to 60-70% over the last three decades (Du *et al.*, 2015; Mintz *et al.*, 2005;). Despite this improvement, the disease has a high recurrence rate, frequently metastasizing to other body parts, particularly the lungs (Janani *et al.*, 2022; Li *et al.*, 2016). Studies indicate that the survival rates post-metastasis range from 20-30% (Durfee *et al.*, 2016). However, even with numerous recent studies, the metastasis mechanism remains incompletely understood (Li *et al.*, 2016).

The current management of osteosarcoma relies on surgical, chemotherapeutic, and radiotherapeutic interventions (Kazantseva *et al.*, 2022; Li *et al.*, 2016). Nevertheless, it is widely acknowledged that osteosarcoma displays poor responsiveness to chemotherapy and has a notable incidence of recurrence (Bernardini *et al.*, 2018; Mintz *et al.*, 2005). Despite chemotherapy treatments, metastatic rates have remained unaltered over the last two decades (Kazantseva *et al.*, 2022). Conversely, researchers have found that drugs used in osteosarcoma treatment have several side effects, including cardiovascular issues, and suppress the immune system (Abdelrheem *et al.*, 2021). In addition, chemotherapy has been reported to induce apoptosis in healthy cells (Majeed *et al.*, 2022; Wang *et al.*, 2020) While the occurrence of

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osteosarcoma is infrequent, reports suggest the necessity for novel drugs and approaches in addressing this ailment due to various factors, including recurrence following treatment, aggressive metastasis, and inadequate response to chemotherapy (Bernardini *et al.*, 2018; Janani *et al.*, 2022; Kumar *et al.*, 2014). Tarkang *et al.* (2015) recommended that plant-based treatments used in complementary medicine could be integrated with surgical and chemotherapeutic regimens in cancer treatment. Additionally, according to Prakash *et al.* (2007), plants constitute a valuable alternative agent source with minimal or no side effects. Kim and Gilbert (2019) claimed that extracts obtained from plants with high levels of secondary metabolites possessing antioxidant properties could aid in preventing cancer or supporting its treatment. According to Kamran *et al.* (2022), terpenoids, which are secondary metabolites, exhibit synergistic effects specifically against cancer cells that have multidrug resistance when paired with cancer drugs such as doxorubicin, cisplatin, paclitaxel, sorafenib, and 5-fluorouracil. Hence, certain terpenoids hold the potential to enhance traditional chemotherapy's response. Additionally, the World Intellectual Property Organization (WIPO) patent database registers 23 patents related to terpenoids' anticarcinogenic potential in treating various types of cancer. Chai *et al.* (2015) noted that while edible macrophytes have not been studied as thoroughly as terrestrial plants, recent research has shown that they have valuable anticancer properties and represent an untapped natural resource for further study. Macrophytes are photosynthetic organisms visible to the naked eye that live in aquatic environments (Justin *et al.*, 2022; Nassouhi *et al.*, 2018). This category includes macroalgae and bryophytes such as liverwort, moss, fern, and angiosperms (Rejmankova, 2011; Unadkat & Parikh, 2021). Studies with aquatic macrophytes have found that extracts from *Pistia stratiotes* and *Eichhornia crascipes* have cytotoxic effects on bone, lung, breast, and prostate cancers (Abraham *et al.*, 2014; Kumar *et al.*, 2014).

Lemna minor is a freshwater floating perennial aquatic plant of the family Lemnaceae (Klaus *et al.*, 2013; Petrova Tacheva *et al.*, 2020). *L. minor* is frequently used in ecotoxicological studies due to its rapid growth, adaptability to laboratory conditions, ease of harvesting, and high biomass production (Alkimin *et al.*, 2019; Ceschin *et al.*, 2020; Khan *et al.*, 2020; Nassouhi *et al.*, 2018; Panfili *et al.*, 2019). Although current studies focus mainly on the removal of heavy metals and toxic chemicals, *L. minor* has a wide range of applications, including feed and food production, biofuel and biogas production, and biotechnological research (Bog *et al.*, 2019; Yahaya *et al.*, 2022). In addition to these studies, *L. minor* is a plant that has been widely used in traditional medicine and homeopathy for many years (Petrova-Tacheva *et al.*, 2019). *L. minor* is known to be used externally as an antipyretic, diuretic, and anti-inflammatory for upper respiratory tract and chronic rheumatic diseases, as well as for eczema, acne, wound healing, and insect bites (Al-Snafi, 2019). Many commercial products derived from L. minor extract are used to treat allergic asthma, rhinitis and nasal congestion. Lemna species contain phenolic compounds such as gallic acid, tannins, flavonoids, anthocyanins, and quercetin, and compounds known as steroids such as thiols and terpenes, suggesting that they may have antimicrobial, antioxidant, and even anticarcinogenic properties (Gonzalez-Renteria *et al.* 2020). Many studies have shown that *L. minor* is an effective antimicrobial (Dafalla, 2015; Gonzalez-Renteria *et al.*, 2020; Gülçin *et al.*, 2010; Tan *et al.*, 2018) and antioxidant (Gülçin *et al.*, 2010; Iskandar *et al.*, 2019; Kim *et al.*, 2012, Saritha & Saraswathi, 2014, Tran *et al.*, 2021). Despite this information cancer studies on *L. minor* are very limited. Within the scope of this study, the anticarcinogenic activity of *L. minor* extract, extracted by microwave method and dried with a lyophilizer, on Saos-2 was investigated. To understand the mechanism of the observed activity, the expressions of bax, bcl-2, caspase-3, and caspase-8 genes were determined by real-time PCR

2. MATERIAL and METHODS

The study was carried out at the Life Sciences Application and Research Center Laboratories of Gazi University (Microbiology, Molecular Biology, and Chemistry Research Laboratories).

The cytotoxicity study was conducted to analyze the efficacy of *L. minor* extracts on cancer cells. Subsequently, RT-PCR was employed to determine the pathway and gene expression induced by *L. minor* extracts with anticarcinogenic activity in the apoptosis study.

2.1. Development and Identification of *L. minor*

The *L. minor* used in the study was obtained from an aquarium store and cultivated at the Hydrobiology Laboratory in the Biology Department of Ankara University. The macrophyte was identified by comparing its morphological features to those described in Güner and Ekim (2014). *L. minor* was cultured in 40% Hoagland medium under a 12:12 light: dark photoperiod at 19.3±2.1 °C, as reported by Ergönül *et al.* (2019).

2.2. Plant Extraction

The plants were harvested and washed with distilled water to eliminate any environmental contaminants. After washing, the plants were air-dried on blotters under normal conditions. Dried *L. minor* was then powdered using a porcelain mortar. An optimization study determined the ideal conditions for extracting phenol from the plants. The study revealed that the highest phenol concentration was achieved at 850 watts, 60% ethanol, and 90 seconds. These conditions were used consistently throughout the study (Dogan *et al.*, 2022). To extract the phenol, one gram of the dried plant sample was mixed with 20 milliliters of 60% ethanol (99.5% Merck, Germany) and subjected to extraction using a microwave device at 850 watts for 30 seconds, done in three runs. The resulting extracts were separated by centrifugation at 2500 revolutions per minute for 20 minutes and filtered through Whatman No. 1 filter paper placed in the upper phase (Yağcıoğlu, 2015). The extracts were then freeze-dried in a lyophilizer (Christ, Alpha 1-2) and stored at +4˚C until analysis (Dogan *et al.*, 2022; Karami *et al.*, 2015).

2.3. Cell Lines

The research utilized a human bone cancer cell line (Saos-2 ATCC HTB-85) and normal human osteoblast (hFOB ATCC CRL-3602) as control. These cell lines were purchased from the American Type Culture Collection (ATCC). The cells stored in liquid nitrogen were thawed in a 37°C water bath containing 10% ice and transferred to the cell medium. To determine the number of viable cells, 1 mL cell suspension was prepared and centrifuged. The obtained pellet was stained with trypan blue to distinguish between live and dead cells, with dead cells appearing blue. The trypan blue-cell mixture was placed on a Thoma slide and live cells were counted under an inverted microscope, according to Doğan (2011).

Cells were grown in RPMI 1640 medium (Capricorn, Germany) containing 1% penicillin/streptomycin antibiotic (Capricorn, Germany) and 10% fetal bovine serum (FBS, Capricorn, Germany) at 37°C in a 5% carbon dioxide atmosphere. The medium was changed every 2 days. Cells showing 80-90% spreading in the flasks were removed with trypsin/EDTA and counted (Doğan, 2023; Morita *et al.*, 2002, Uçar *et al.* 2016).

2.4. Investigation of Cytotoxicity

In 96-well plates, $2x10^5$ cells were transferred to each well and allowed to grow. *L. minor* extract was dissolved in a cell medium. Concentrations of 5000-10 µg/mL were prepared by serial dilution (Agan *et al.*, 2020). The prepared samples were applied to developing healthy and cancer cells and cultured for 12 and 24 hours. At the end of the incubation, the cytotoxicity level was measured by colorimetric evaluation using the MTT kit (Biotium, USA). 20 µL MTT was added to all wells and incubated at 37°C for 3 hours. After 3 hours, the samples in the wells were removed, and the dye was dissolved with DMSO and measured at a wavelength of 570 nm (Epoch, BioTek). Cells without extract were used as a control. The control was considered 100% viable and % inhibition and IC_{50} values were calculated accordingly (Kaya *et al.*, 2024; Luca *et al.*, 2022).

2.5. Identification of Apoptotic Cells

IC_{50} values for the *L. minor* extract were obtained through cytotoxicity analysis. The mRNA expression levels of genes involved in the regulation of apoptosis, bax, bcl2, caspase3, and caspase8, were measured by quantitative real-time PCR (qPCR) (Janani *et al.*, 2022; Wang *et al.*, 2020). RNA was extracted from cells treated with plant extract and after cDNA synthesis, the PCR products were labeled with SYBR green dye (Kuang *et al.*, 2018).

2.5.1. *Total RNA isolation*

Total RNA isolation was performed using the Hybrid-R kit protocol (GeneAll, South Korea). The extracted RNA was quantified and evaluated for purity using Qubit4 (Invitrogen). Samples with an absorbance range of A_{260}/A_{280} ratio between 1.8-2.0 were chosen to initiate complementary DNA (cDNA) synthesis (Kuang *et al.*, 2018).

2.5.2. *cDNA synthesis*

The cDNA synthesis of isolated and quantified mRNA samples abided by the protocol of the cDNA Synthesis Kit (ABT, Wizbio WizScript™ W2211). To begin with, 10 µL of the sample was added to the mixture outlined in Table 1 before undergoing cDNA synthesis in the Applied Biosystems Veriti heat cycler model, following the parameters depicted in Table 1. Eventually, the cDNA samples were frozen at -20°C until carrying out the qPCR study.

Table 1. cDNA synthesis mixture and conditions.

Mixture	Volume (µL)
20X dNTP mix	1
WizScript™ RTase	1
RNase free water	3.5
10X reaction buffer	2
Random hexamer	2
RNase Inhibitor	0.5
RNA Sample	10
Total	20

Temperature (°C)	Time (Minute)	Cycle
25	10	1
37	20	6
85	5	1
4	∞	∞

2.5.3. *Determination of gene expression by Real Time PCR*

Changes in the expression of bax, bcl-2, caspase-3, and caspase-8 genes in Saos-2 cells treated with *L. minor* extract compared to untreated cells were evaluated using RT-PCR (Applied Biosystems, 7500 Fast Real-Time PCR). The PCR reaction mixture contained 12.5 µL of 2x SYBR Green qPCR master mix, 1 µL of cDNA, 2 µL of primer mix (10 µM stock solutions), and 9.5 µL of PCR grade water. RT-PCR was programmed by following conditions for one cycle: 95 °C for 10 min and 40 cycles: 95 °C for 10 s and 60 °C for 30 s. Analysis of the expression levels of the genes was carried out using the $2^{-\Delta\Delta Ct}$ method. The ACTB gene was determined as a housekeeping gene. Primers are listed in Table 2.

Table 2. Primers used to determine gene expression.

Primer	Primer Sequence
BAX-f	GATGGACGGGTCCGGGG
BAX-r	CGATCCTGGATGAAACCCTGA
BCL2-f	GGATAACGGAGGCTGGGATG
BCL2-r	TGACTTCACTTGTGGCCCAG
CASP3-f	ATTTGGAACCAAAGATCATACATGG
CASP3-r	TTCCCTGAGGTTTGCTGCAT
Caspase 8-f	GCGGAGGGTCGATCATCTAT
Caspase 8-r	CACAACTCCTCCCCTTTGCTG
ACTB-F	CATCCTCACCCTGAAGTACC
ACTB-R	TGAAGGTCTCAAACATGATCTG

3. RESULTS

3.1. Investigation of Cytotoxicity

Saos-2 cells were cultured and transferred to 96-well plates at 80% confluence. Time and concentration experiments were conducted using *L. minor* extract concentrations ranging from 0-5000 µg/mL, and cytotoxicity was observed at 12 and 24 hours. At 12 hours, no cytotoxic effects were observed at initial concentrations of 10, 20, 40, and 78 µg/mL, but effectiveness was observed at concentrations of 156 µg/mL and above. Cytotoxicity was detected at all concentrations within 24 hours of applying the extract. The highest mortality rate was 79% after 12 hours of application, increasing to 95% after 24 hours. The death rate of cancer cells appears to vary depending on the concentration and duration of treatment, with greater concentrations resulting in increased cancer cell death (Figure 1).

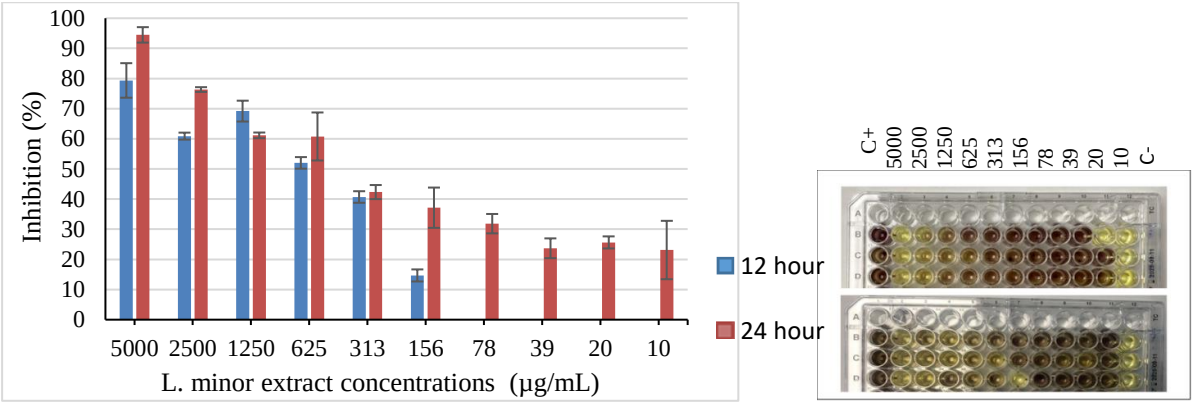

Figure 1. Inhibitory effect of *L. minor* accumulations on bone cancer cell line (Saos-2) at 12 and 24 hours. C+(Positive control): extract-free cells, C-(Negative control): medium without cells and extracts.

Macrophytes with cytotoxic effects on cancer cells should lack cytotoxicity on healthy cells. For control purposes, this study utilized the hFOB cell line, a healthy human epithelial cell. The results demonstrated that low extract dosages generally did not harm healthy cells, but rather enhanced cell growth. Furthermore, at a concentration of 313 µg/mL, *L. minor* extract exhibited a 42% cancer cell mortality rate, yet increased the growth of healthy cells by 25%. At this concentration, *L. minor* did not harm the healthy cells or increase their development. However, when the concentration exceeded 625 µg/mL, the extracts were observed to cause cell inhibition. The results indicate that the extracts are non-toxic, with IC_{50} values below the inhibitory concentration of 625 µg/mL (Figure 2).

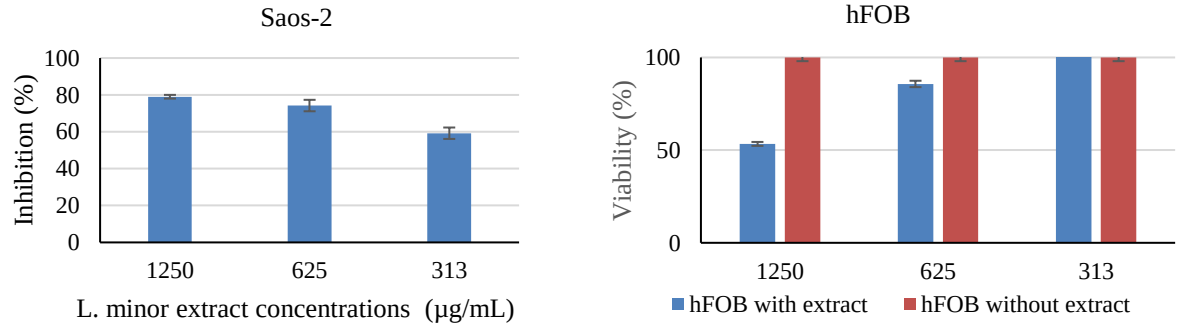

Figure 2. Effect of *L. minor* concentrations in the IC_{50} range on cells A. Saos2: cancer cell, B. hFOB: healthy cell at 24 h.

3.2. Identification of Apoptotic Cells

In this study**,** compared with control cells, the expression of bax, caspase-3, and caspase- 8 *L. minor* treated groups was significantly increased, whereas the level of bcl-2 was decreased (Figure 3).

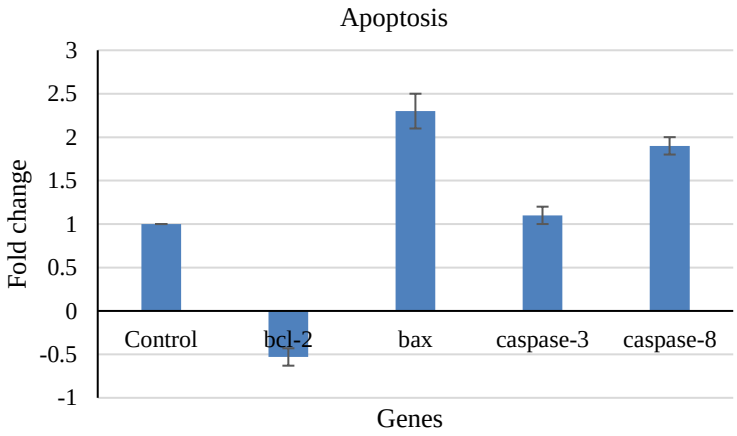

Figure 3. Bcl-2, bax, caspase-3, and caspase-8 expression.

4. DISCUSSION and CONCLUSION

Cancer is the second leading cause of death worldwide (Abdelrheem *et al.*, 2021; Janani *et al.*, 2022). Osteosarcoma, a type of malignant bone cancer that mainly affects children and young adults aged 0-24 years, accounts for 5% of all childhood cancers (Majeed *et al.*, 2022). Although osteosarcoma is rare, there is an urgent need for alternative medicines and new approaches due to factors such as treatment relapse, aggressive metastasis, and poor response to chemotherapy (Bernardini *et al.*, 2018; Janani *et al.*, 2022; Kumar *et al.*, 2014,). Chai et al (2015) have highlighted the potential of macrophytes in cancer research, although there has been a limited amount of research carried out in this area. Abraham *et al.* (2014) reported that the hexane and methanol extracts from *P. stratiotes* leaves caused a 60% inhibition on human bone cancer MG 63. Bernardini *et al.* (2018) investigated the effect of *Padina pavonica* algae extracts on human bone cancer Saos-2 and MNNG. As a result of the study, they determined that the extract had a cytotoxic effect on both cell lines. In the proliferation study, they identified a significant decrease in Saos-2 proliferation - by 70% - when an IC_{50} dose of the extract applying. In a different study, it was determined that there was almost 90% inhibition of the human bone cancer U2OS cell line incubated with *Nymphoides peltata* (Little Water Lily) extract obtained with 10% methanol for 48 hours (Du *et al.* 2015). The results of our study indicate that *L. minor* exhibited high cytotoxic activity against the Saos-2 cell line. Cytotoxicity was found to be 79% in 12 hours of incubation and 95% in 24 hours of incubation. In comparison to other studies, it is evident that the rate of killing bone cancer cells is significantly high. This activity is believed to be related to the compounds such as secondary metabolites contained in macrophytes. A body of evidence indicates that antioxidants and anticarcinogenic effects are linked, with phenolic compounds playing a pivotal role in cancer prevention (Janani *et al.*, 2022). The phytochemical content of *L. minor* used in this study was analyzed by GC-MS in previous studies. It was found that *L. minor* extract has a vibrant content, especially in phytol, thymol, hexanal, 2,4-di-tert-butylphenol, 5-tetradecane, cetene (Dogan *et al.*, 2022). These compounds are thought to contribute to the high anticancer activity of our plant.

Despite numerous studies conducted in recent years, the mechanism underlying the development and metastasis of osteosarcoma is still not fully understood. Nevertheless, the consensus of these studies is that apoptosis in osteosarcoma is related to bcl-2/bax balance or caspase activation (Imran *et al.*, 2021; Wang *et al.*, 2020). Wang *et al.* (2020) also suggest that apoptosis in osteosarcoma occurs through a mitochondria-dependent pathway with increased pro-apoptotic bax expression and decrease bcl-2 expression. Recent studies have shown that metabolites obtained from plants induce human cancer cells to apoptosis. Janani *et al.* (2022) reported that certain plant extracts, such as Merremia emarginata caused apoptosis in Saos-2 by increasing bax expression and decreasing bcl-2 expression. Kamran *et al.* (2022) stated that terpenoids such as phytol, thymol, carvacrol, and thymoquin derived from plants induced the

death of human cancer cells such as Saos-2, MG 63 by using caspase-9, caspase-3 pathways and regulating bax/bcl-2 balance. Similarly, Zhu (2022) showed that pseudolaric acid B (PAB) a natural diterpenoid, down-regulated the expression of Bcl-2 and pro-caspase-3 and increased the expression of bax and caspase 3, leading to human bone cancer (MG 63) to apoptosis via the mitochondrial pathway. In this experiment, the human osteosarcoma cell line Saos-2 was first treated with *L. minor* extract. The expression of bax and caspase-8 was significantly increased in the *L. minor* extract groups, whereas the level of bcl-2 was decreased when compared with the control cell. In our study, after the treatment of L. *minor* extract, the cell viability was decreased. Our study results revealed that *L. minor* extract directs cancer cells to apoptosis through a mitochondria-dependent pathway maintaining the bax/bcl-2 balance. A significant increase in caspase 8, the initiator gene of apoptosis, was observed in cells treated with *L. minor*. However, no significant increase was observed in caspase-3, which is considered a lethal caspase. Caspase-8 is responsible for the initiation of apoptosis via the extrinsic pathway. Activated caspase-8 activates lethal caspase-3/caspase-7, leading the cancer cell to apoptosis (Boice, A., and Bouchier-Hayes, 2020). It is thought that *L. minor* initiates apoptosis with caspase 8, but may subsequently activate caspase-7 instead of the studied caspase-3. The results we obtained showed that our study is compatible with previous similar studies.

The high rate of recurrence and metastasis of bone cancer, coupled with the fact that chemotherapy also damages healthy cells, underscores the necessity of integrating traditional treatments with chemotherapy and drugs in bone cancer treatments. The study demonstrated that the *L. minor* macrophyte had a lethal effect on the human bone cancer Saos-2 cell line, but did not harm healthy human cells. These findings indicate that *L. minor* has potential as a treatment for osteosarcoma.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Research Article

Antifungal activity of extracts from *Ulva*, *Sargassum*, and *Gracilaria* against three fungal pathogens and GC-MS analysis of the most effective extracts

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Abstract: Coastal marine macroalgae are highly diverse and rich in bioactive compounds, though only a few studies have explored their antifungal potential against plant pathogens in Sri Lanka. This study investigated the antifungal activity of *Ulva* sp., *Gracilaria* sp., and *Sargassum* sp. from Thalpe Reef, Galle, Sri Lanka, against the fungal pathogens *Lasiodiplodia theobromae, Pseudopestalotiopsis theae,* and *Diaporthe eugeniae*. These pathogens cause leaf necrosis, leaf chlorosis, and leaf blight, respectively, in *Solanum melongena* plants. To evaluate the antifungal activity of each species, sequential crude extraction was performed using ethyl acetate and methanol. The poisoned food technique was used to screen the antifungal activity and extracts showing the highest antifungal activity were further analyzed using Gas Chromatography-Mass spectrometry (GC-MS). The best inhibition against *D. eugeniae* and *P. theae* was exhibited by *Ulva*-ethyl acetate (UE) at 2000 ppm with inhibition percentages of 79.29% and 56.68%, respectively. *Ulva*-methanol (UM) at 2000 ppm showed the highest inhibition against *L. theobromae*, with an inhibition percentage of 43.09%. These results revealed that UE and UM extracts effectively controlled tested fungal pathogens. GC-MS analysis revealed the presence of three compounds in UE, nine in UM, and seven in *Gracilaria*-ethyl acetate (GE) extracts. Notably, the most abundant compounds with potential antifungal activity included Dihydroactinidiolide (30.02%), 4-Hydroxy-2-butanone (37.37%), and 6,10,14-Trimethylpentadecan-2-one (58.86%).

1. INTRODUCTION

Marine macroalgae, or seaweeds, are multicellular, eukaryotic, photosynthetic organisms (Makkar *et al*., 2016). They are classified into three divisions based on pigmentation: Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae) (Biris-Dorhoi *et al*., 2020). By 2009, 125 macroalgal taxa including 44 Chlorophyceans, 10 Phaeophyceans, and 71 Rhodophyceans, had been identified along the Sri Lankan coast. The Thalpe Reef in Sri Lanka features an extensive coastline with a diverse population of marine macroalgae, where *Ulva*, *Sargassum*, and *Gracilaria* are the most abundant genera for their respective divisions (Coppejans *et al*., 2009).

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Marine algae are utilized as food sources for both humans and animals, as fertilizers, biofuels, and raw materials for industrial products such as agar, carrageenan, and alginate (Jayasinghe *et al.*, 2018). This is due to their high content of complex organic compounds, alongside primary and secondary metabolites with diverse biological activities. The bioactive chemicals in macroalgae include carbohydrates, peptides, lipids, enzymes, vitamins, phytopigments (xanthophylls and carotenoids), phenolic compounds, tannins, and terpenoids (Biris-Dorhoi *et al.*, 2020). These compounds exhibit various pharmacological activities, such as anticancer, antioxidant, antimicrobial, antifungal, antiviral, and anti-inflammatory effects (Pérez *et al*., 2016). The extent of their antifungal and antioxidant effect is generally attributed to their phenolic compositions (Jayaprakasha *et al*., 2003).

Plant diseases significantly impact crop yield, with over US$ 220 billion spent annually on disease management practices (FAO, 2022). These diseases can be caused by various agents, including bacteria, viruses, nematodes, parasitic plants, and especially fungi. Currently, farmers heavily rely on chemical pesticides and fertilizers to boost crop production. According to Padmajani *et al*. (2014), herbicides are the most commonly used pesticides in Sri Lanka followed by insecticides. The vegetable sector heavily relies on insecticides, with fungicides being the second most prevalent (Nagenthirarajah & Thiruchelvam, 2008). In 2011, Sri Lanka imported 8902.87 metric tons of pesticides, a 49% increase from 2006 (Padmajani *et al*., 2014). Further, significant concentrations of organo-chlorine and organophosphate pesticides have been detected in the Walawe and Nilwala rivers (De Silva, 2003). Also, it is estimated that more than 50% of pesticides do not reach their target and instead contaminate the soil (Padmajani *et al*., 2014). The improper use of pesticides leads to immediate health effects as well as long-term health risks such as cancer, kidney ailments, and reproductive problems. Furthermore, in Sri Lanka, farmers who apply pesticides are at a higher risk of developing chronic renal failure (Wanigasuriya *et al*., 2007). Consumer attitudes toward pesticide use in agriculture have shifted due to these health impacts and environmental damage, increasing demand for safer and more efficient alternatives (Aktar *et al*., 2009). Marine resources, including algal species, offer a vast reservoir of unique, biologically active compounds. These species thrive under extreme climatic and environmental stresses such as high salinity, intense light, and high temperatures, making them potential sources for discovering novel and effective compounds for plant disease management (Maldeniya *et al.,* 2020).

Few studies have investigated the functional properties of Sri Lankan macroalgal extracts, particularly their antifungal properties against plant pathogens. Therefore, this study aims to determine the antifungal potential of ethyl acetate and methanol extracts from *Ulva* sp., *Sargassum* sp., and *Gracilaria* sp. against the fungal pathogens *Diaporthe eugeniae*, *Pseudopestalotiopsis theae* and *Lasiodiplodia theobromae*, which affect *Solanum melongena*. The poisoned food technique was used to assess antifungal activity, and Gas Chromatography-Mass Spectrometry (GC-MS) analysis was employed to identify the potential antifungal compounds present in the algal extracts.

2. MATERIAL and METHODS

2.1. Sample Collection and Preparation

2.1.1 *Collection of macroalgae samples*

Samples of *Ulva* sp., *Sargassum* sp., and *Gracilaria* sp. were collected from Thalpe Reef (6.00^{o} N, 80.29^{o} E) in Galle, Southern Province of Sri Lanka, based on their abundance in September 2022 (Figure 1). The algal samples were hand-picked and placed in zip-lock bags half-filled with seawater for transport to the laboratory at the University of Kelaniya, Sri Lanka. Initially, the samples were washed with seawater to remove sand particles, invertebrates, and epiphytes, followed by a rinse with tap water to eliminate salt. The algal samples were identified based on macroscopic and microscopic morphological characteristics as described by Durairatnam (1961) and Coppejans *et al*. (2009). The algal samples were then air-dried for approximately ten days and ground into a fine powder using an electric blender. The powdered samples were stored in sterilized glass bottles and algal extracts preparation was started on the same day.

Figure 1. Thalpe Reef, Sri Lanka.

2.1.2 *Preparation of macroalgal extracts*

Bioactive compounds in 7.0 g of dried powder from each macroalgal sample were sequentially extracted using 150 mL of ethyl acetate in a Soxhlet apparatus (Electrothermal, Canada) for four hours, followed by extraction with an equal amount of methanol (Martins *et al*., 2018). The heating mantle temperature of the Soxhlet apparatus was kept below the boiling points of each solvent (Radhika & Mohideen, 2015). Organic solvents were evaporated under reduced pressure using a rotary evaporator (BIOBASE, RE-201D, China) at 35 rpm and 40 ^{o}C to obtain the crude extracts, which were then stored in a refrigerator at 4 ^{o}C until further use. The percentage yield (% yield) of the crude product was determined using the equation provided by Agbaje-Daniels *et al*. (2020).

$$\% \text{ yield } = \frac{\text{Weight of the crude (g)}}{\text{Weight of the dried algae powder used for extraction (g)}} \text{ x } 100$$

2.1.3 *Preparation of fungal cultures*

Fungal cultures of *Diaporthe eugeniae* U11 (MT990529), *Pseudopestalotiopsis theae* U10 (MT990526) and *Lasiodiplodia theobromae* H32A (MT990527), isolated from *Solanum melongena* (brinjal) leaves showing symptoms of leaf blight, leaf yellowing and leaf necrosis, respectively, were obtained from the Department of Plant and Molecular Biology at the University of Kelaniya. Mycelial discs (5 mm) were cut using a cork borer and aseptically transferred onto petri plates containing potato dextrose agar (PDA). Petri plates were incubated at room temperature (30±2 ^{o}C).

2.2 Screening of Macroalgal Extracts for Antifungal Activity

The poisoned food technique was performed as described by Abhishek *et al*. (2021). Four concentrations of algal extract (250 ppm, 500 ppm, 1000 ppm, and 2000 ppm), were incorporated into PDA plates. The required weight of crude algal extract for each concentration was measured and dissolved in 200 µL of dimethyl sulfoxide (DMSO). The dissolved crude was then mixed into melted PDA medium (40 ^{o}C) in a conical flask. Fifteen mL of the agar-crude extract mixture was poured into petri plates (poisoned plates) and allowed to solidify.

Agar disks containing the fungus (5 mm diameter) were cut using a cork borer from the peripheral regions of seven-day-old cultures of *D. eugeniae, P. theae* and *L. theobromae,* and transferred to the center of the poisoned agar plates. Five replicates were prepared for each experiment. Additionally, three negative controls were prepared by adding equal amounts of 0.03% (v/v) DMSO without algal extracts, and three positive controls were prepared with Captan (a commercial fungicide) at a concentration of 1000 ppm. Plates with *D. eugeniae* and *P. theae* were incubated at room temperature (30±2 ^{o}C) for seven days, while plates with *L. theobromae* were incubated at the same temperature for 24 hours. After the incubation period, the radial growth of the fungal colony (diameter in mm) was measured using a ruler along two perpendicular axes and the average diameter was calculated. The inhibition percentage of

fungus was calculated using the following equation based on the average radial growth (Ammar *et al.*, 2017).

$$I\ \% = (C-T)/C \times 100$$

Where, I % = inhibition percentage, C = radial growth in control DMSO plates, T = radial growth in plates with each concentration of crude extract

2.3 Statistical Analysis

All the data were presented as mean values ± standard error. The Kruskal-Wallis test and Dunn's test were used for the statistical analysis of percentage inhibition data using R software (version 4.3.3).

2.4 GC-MS Profiling of Selected Macroalgal Extracts

The *Ulva*-ethyl acetate (UE), *Ulva*-methanol (UM), and *Gracilaria*-ethyl acetate (GE) extracts subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis as described by Kamal *et al*. (2011) at the Residue Analysis Laboratory, Industrial Technology Institute (ITI), Colombo 7, Sri Lanka, to identify compounds potentially possessing antifungal properties. The samples were analyzed using a GC system (Agilent, 6890 series) equipped with an HP-5 MS column (0.25 mm x 30 m x 0.25 µm), with Helium serving as the carrier gas at a flow rate of 1.0 mL/min. The injector volume was 0.2 µL, with injector and detector temperatures set at 250 ^{o}C and 300 ^{o}C, respectively. The oven temperature was initially maintained at 40 ^{o}C for 5 minutes, then increased to 240 ^{o}C at a rate of 15 ^{o}C/min, followed by a further increase to 280 ^{o}C for 2 minutes at a rate of 10 ^{o}C/min.

Peak identification was conducted by comparing the obtained mass spectrum with the National Institute of Standards and Technology (NIST) mass spectral library (Shobier *et al.*, 2016). To assess the antifungal potential, the identified compounds were analyzed using the PASS (Prediction of Activity Spectra for Substances) online Program using Way2Drug informational-computational platform (version 2.0) (Chy *et al*., 2019; Druzhilovskiy *et al.*, 2017).

3. RESULTS

3.1. Yield of Macroalgal Crude Extracts Using Methanol and Ethyl Acetate

The choice of organic solvent in the extraction process significantly influenced the extract yield (p=0.033). Methanol produced the highest yield across all algal species, with yields of 2.01%, 2.61%, and 3.21% of the total weight for *Ulva* sp., *Sargassum* sp., and *Gracillaria* sp., respectively (Figure 2).

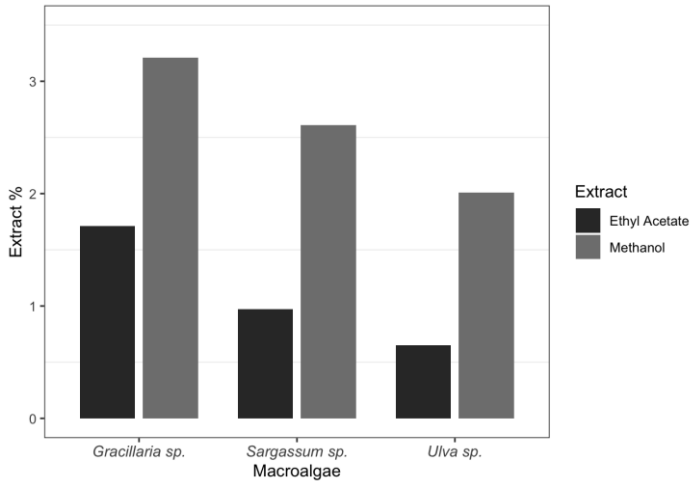

Figure 2. Percentage yield of crude extracts obtained from *Ulva* sp., *Sargassum* sp., and *Gracillaria* sp. with methanol and ethyl acetate.

3.2 Screening macroalgal extracts for antifungal activity

The inhibition percentage of *D. eugeniae* (p=2.61e^{-14}), *L. theobromae* (p=1.88 e^{-13}), and *P. theae* (p=6.42e^{-14}) by the three macroalgae were statistically significant at 0.05 confidence level.

The highest inhibitory percentage against *D. eugeniae* was demonstrated by the UE extract at 2000 ppm, reaching up to 79.29% (Table 1, Figure 3). The lowest inhibition was shown by the *Sargassum*-methanol extract (SM) at 1000 ppm, with only 0.81%. SE extracts at 250 ppm and 500 ppm were ineffective in inhibiting *D. eugeniae* growth. The UE extract exhibited the best antifungal properties against *D. eugeniae*. The GE extract, showing more than 50% inhibition at its lowest concentration (250 ppm), can also be considered an effective antifungal extract. The highest inhibition percentage against *L. theobromae* was shown by the UM extract at 2000 ppm (43.09%), followed by the *Gracilaria*-methanol (GM) extract at 2000 ppm (41.18%), and 1000 ppm (40.83%) (Table 1, Figure 3). The lowest inhibition was shown by the GE extract at 500 ppm (01.53%). None of the SM extracts exhibited any antifungal activity against *L. theobromae*.

For *P. theae*, the inhibitory percentages of each algal extract and concentration were significantly different (Table 1, Figure 3). The maximum inhibitory percentage against *P. theae* was recorded with UE extract at 2000 ppm (56.68%), although there was no significant difference compared to the UE extract at 1000 ppm (53.16%) and at 500 ppm extract (50.08%). The minimum inhibition was observed with the GM extract at 2000 ppm (05.20%).

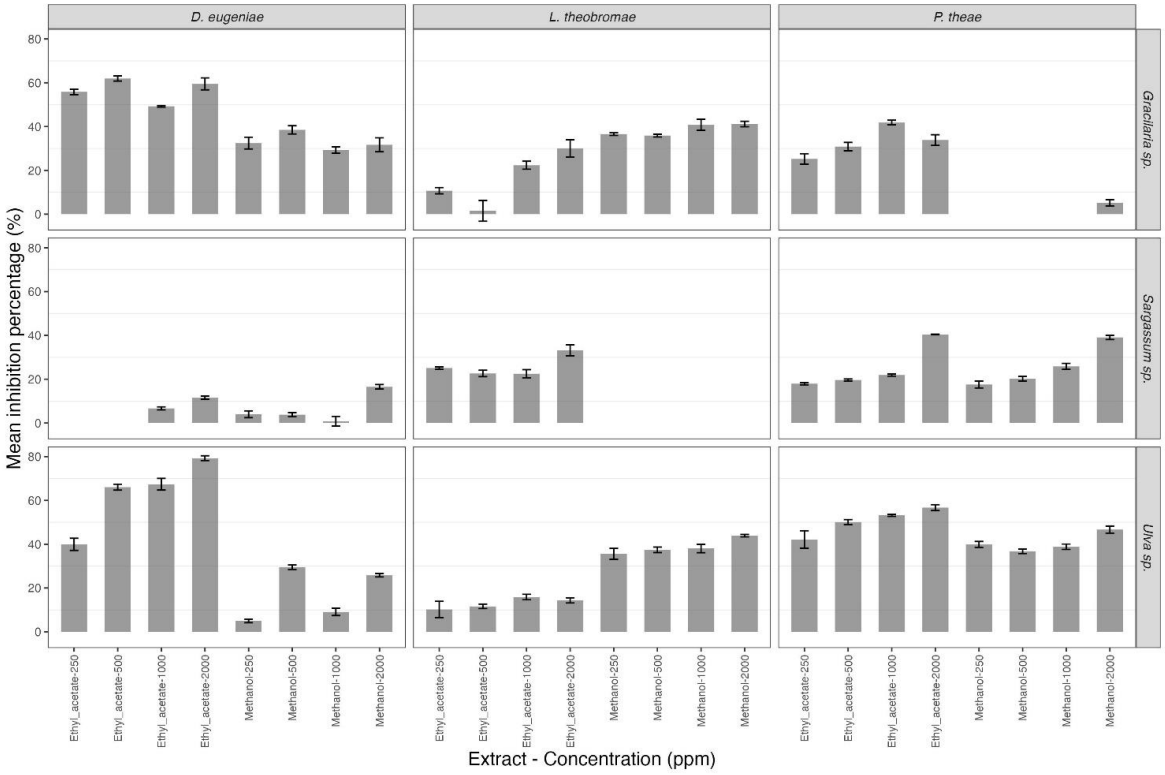

Figure 3. The graph of inhibition percentages of *Ulva* sp., *Sargassum* sp., and *Gracilaria* sp. ethyl acetate and methanol extracts against *D. eugeniae*, *L. theobromae* and *P. theae*

3.3 GC-MS profiling of selected macroalgal extracts

The most effective antifungal extracts were subjected to GC-MS analysis to identify the chemical compounds with antifungal potential. In the GE extract seven different peaks were identified, indicating the presence of seven chemical compounds. Similarly, three potential chemical compounds were identified in the UE extract, while nine were found in the UM extract. These compounds underwent evaluation for antifungal activity using previous literature (Abbassy *et al*., 2014; Johnson *et al.*, 2014; Shobier *et al.*, 2016; Ragunathan *et al*., 2019) and the PASS online program (Table 2). According to PASS predictions, few identified chemical compounds demonstrate promising antifungal activity. Notably, the most abundant compounds with antifungal activity in the GE, UM, and UE extracts were 6,10,14-Trimethylpentadecan-2-one (58.86%), 4-Hydroxy-2-butanone (37.37%), and Dihydroactinidiolide (30.02%), respectively.

Table 1. Inhibition percentages of *Ulva* sp., *Sargassum* sp., and *Gracilaria* sp. methanol and ethyl acetate extracts against *D. eugeniae*, *L. theobromae*, and *P. theae*.

Algae	Solvent	Concentration (ppm)	Inhibition percentage (%) ± Standard Error		
			D. eugeniae	*L. theobromae*	*P. theae*
Ulva sp.	Ethyl acetate	250	39.94^{abcde} ± 2.76	10.19^{abc} ± 3.71	42.17^{abcd} ± 3.96
		500	66.03^{cd} ± 1.35	11.61^{abc} ± 1.02	50.08^{bcd} ± 1.15
		1000	67.38^{cd} ± 2.67	15.87^{abc} ± 1.25	53.16^{bd} ± 0.46
		2000	**79.29^{d} ± 1.10**	14.34^{abc} ± 1.19	56.68^{d} ± 1.35
	Methanol	250	04.95^{abe} ± 0.80	35.62^{abc} ± 2.49	**39.90^{abcde} ± 1.35**
		500	29.49^{abcde} ± 1.04	37.42^{ac} ± 1.26	36.76^{abcde} ± 1.01
		1000	09.09^{abcde} ± 1.68	38.03^{ac} ± 1.93	38.84^{abcde} ± 1.24
		2000	25.8^{abcde} ± 0.78	**43.09^{c} ± 0.55**	46.69^{bcd} ± 1.62
Sargassum sp.	Ethyl acetate	250	-	25.11^{abc} ± 0.54	17.92^{acd} ± 0.51
		500	-	22.62^{abc} ± 1.46	19.62^{abce} ± 0.46
		1000	06.68^{abce} ± 0.68	22.50^{abc} ± 1.85	21.89^{abcde} ± 0.48
		2000	11.52^{abcde} ± 0.73	33.20^{abc} ± 2.48	40.44^{abcde} ± 0.11
	Methanol	250	03.97^{abe} ± 1.55	-	17.57^{ace} ± 1.58
		500	03.78^{abe} ± 0.94	-	20.19^{abcde} ±1.08
		1000	00.81^{be} ± 2.21	-	25.86^{abcde} ± 1.37
		2000	16.53^{abcde} ± 1.01	-	39.06^{abcde} ± 0.97
Gracilaria sp.	Ethyl acetate	250	55.83^{abcd} ± 1.25	10.73^{abc} ± 1.43	25.22 ^{abcde} ± 2.38
		500	61.94^{acd} ± 1.16	01.53^{ab} ± 4.74	30.86 ^{abcde} ± 1.90
		1000	49.25^{abcde} ± 0.35	22.35^{abc} ± 1.82	41.85^{abcd} ± 1.06
		2000	59.51^{acd} ± 2.72	30.06^{abc} ± 3.98	33.84^{abcde} ± 2.44
	Methanol	250	32.47^{abcde} ± 2.68	36.57^{abc} ± 0.61	-
		500	38.51^{abcde} ± 1.87	35.88^{abc} ± 0.65	-
		1000	29.30^{abcde} ± 1.48	40.83^{ac} ± 2.51	-
		2000	31.69^{abcde} ± 3.20	41.18^{c} ± 1.25	05.20^{ae} ± 1.41
Positive control		1000	94.30 ± 0.80	91.17 ± 0.76	86.66 ± 0.92

Note: Means with different letters within a column are significantly different (p=0.05); Negative control data were incorporated into calculations according to formula in Method 2.2.1.

Table 2. Chemical compounds exhibiting antifungal properties from *Ulva*-ethyl acetate, *Ulva*-methanol and *Gracilaria*-ethyl acetate extracts identified through GC-MS analysis and their probabilities of antifungal activity determined using PASS WAY2DRUG online software.

Algal extract	Compound	Compound CID	Molecular formula	Retention time Ttime (min)	% of total	Chemical group	Pa	Pi
Ulva-ethyl acetate	Dihydroactinidiolide	27209	$C_{11}H_{16}O_2$	14.94	30.02	Terpene	0.28	0.09
	Heptadecene	5364555	$C_{17}H_{34}$	16.94	12.28	Alkene	0.47	0.04
Ulva-methanol	Phenylephrine 3-Methoxyamphetamine	6041 152234	$C_9H_{13}NO_2$ $C_{10}H_{15}NO$	01.10	00.12	Phenol	0.26	0.10
	4-Hydroxy-2-butanone	111509	$C_4H_8O_2$	01.15	37.37	Ketone	-	-
	17-Octadecenal Bicyclo[3.1.1]heptane,2,6,6 trimethyl-, (1alpha,2beta,5alpha)-	41922 12314300	$C_{18}H_{34}O$ $C_{10}H_{18}$	02.33	01.75	Aldehyde	0.32	0.08
	Palmitic acid	985	$C_{16}H_{32}O_2$	16.80	00.57	Fatty acid	0.59	0.02
Gracilaria-ethyl acetate	6,10,14-Trimethylpentadecan-2-one	10408	$C_{18}H_{36}O$	16.00	58.86	Sesquiterpenoid	0.38	0.06
	Dihydroactinidiolide	27209	$C_{11}H_{16}O_2$	25.68	00.03	Terpene	0.28	0.09
	(E)-5-Octadecene	5364598	$C_{18}H_{36}$	07.00	04.25	Alkene	0.30	0.08
	Heptadecane	12398	$C_{17}H_{36}$	14.94	02.09	Alkene	0.47	0.04

Note: Pa = probability to be active, Pi = probability to be inactive; Pa higher than Pi are considered to possess antifungal potential

4. DISCUSSION

The majority of algae species produce unique secondary metabolites with various biological capabilities, such as antifungal, antibacterial, antiviral, antioxidant, anticancer, and anti-inflammatory effects (Omar *et al.*, 2018). Due to exposure to challenging environmental conditions like salt, light, temperature, and marine chemical composition, most algal species generate distinct secondary metabolites (Mickymaray & Alturaiki, 2018). In Sri Lanka, only a limited number of studies have explored the biological activities of marine macroalgae, with few focusing on the antifungal potential of marine macroalgae against plant-pathogenic fungi (Fernando *et al.*, 2017; Lakmal *et al*., 2014). Therefore, this study investigates the antifungal potential of *Ulva* sp., *Sargassum* sp., and *Gracilaria* sp. found on the Thalpe Reef in Sri Lanka against selected pathogenic fungi of *S. melongena.* The potential antifungal compounds were identified through GC-MS analysis and PASS online server. The chemical components were extracted sequentially using the Soxhlet apparatus with two solvents of increasing polarity; ethyl acetate and methanol. The number and quantity of bioactive compounds dissolved in a solvent mainly depend on its polarity (Ullah *et al.*, 2019).

In *D. eugeniae, Ulva* sp. exhibited a higher inhibitory percentage with ethyl acetate-2000 ppm extract (79.29%), followed by ethyl acetate-1000 ppm extract (67.38%). Methanol-2000 ppm extract resulted in 25.8% inhibition. In *L. theobromae, Ulva* sp. showed a higher inhibitory percentage with methanol-2000 ppm extract (43.09%). For *P. theae, Ulva* sp. demonstrated a higher inhibitory percentage with ethyl acetate-2000 ppm extract (56.68%), followed by methanol-2000 ppm extract (46.69%). Similarly, Bahammou *et al*. (2021) reported that a 2 mg mL^{-1} methanol extract of *Ulva lactuca* exhibited the highest antifungal activity against plant-pathogenic fungi *Botrytis cinerea*, with an inhibition diameter of 9.5±0.07 mm and *Penicillium digitatum*, with an inhibition diameter of 10.1±0.13 mm. Further, Chanthini *et al*. (2012) documented an antifungal effect of 5% ethyl acetate extract of *U. lactuca* against *Alternaria solani* with an inhibition percentage of approximately 35% using the disk diffusion method. Moreover, Mostafa *et al*. (2021) reported antifungal activity of *Ulva fasciata* extracts against the pathogenic fungus *Fusarium solani*, with methanol and ethyl acetate extracts showing inhibition percentages of 4% and 26.8%, respectively. Additionally, Supriya & Haritha (2022) found that the ethyl acetate extract of *U. lactuca* demonstrated antifungal activity against *Aspergillus oryzae* (69.16%), *Rhizopus artocarpi* (37.73%), and *Fusarium oxysporum* (53.65%). Their study also revealed that the methanol extract showed even higher antifungal activity, with inhibition rates of 74.91% against *A. oryzae*, 61.92% against *R. artocarpi*, and 67.68% against *F. oxysporum*.

The findings of this study indicate that, in many cases, ethyl acetate extracts and occasionally methanol extracts exhibit the highest antifungal potential against the studied plant-pathogenic fungi. However, previous studies have often reported that methanol extracts demonstrate the highest antifungal activity. This deviation could be attributed to differences in the secondary metabolites of marine macroalgae, influenced by variations in geographical locations, environmental factors, and maturity stage of the macroalgal specimens. Additionally, in the present study, the algal extractions were conducted sequentially, beginning with ethyl acetate followed by methanol, a greater proportion of bioactive compounds may have been extracted into ethyl acetate solvent. Further, antifungal activity can differ depending on the fungal species or strain being tested. Some fungi may be more sensitive to compounds extracted with methanol, while others may exhibit better responses to compounds extracted with ethyl acetate. Therefore, the differences in fungal strains used in different studies could contribute to the variation in reported results.

The mechanism of action of antifungal compounds derived from macroalgae remains incompletely understood, with several proposed mechanisms. Typically, compounds present in various algal extracts can target fungi by affecting the cell wall or membrane, as well as intracellular organelles such as the nucleus and mitochondria. Upon penetration of the fungal

cell, antifungal agents may disrupt protein synthesis, and interfere with the mitochondrial respiratory chain, thereby disturbing the cell's homeostasis and stability, ultimately reducing its lifespan (Lopes *et al.*, 2013). Fatty acids identified in macroalgae have exhibited antifungal properties by integrating into the fungal membrane, increasing its fluidity and permeability, and inducing changes in its organization, leading to cell death (Avis & Bélanger, 2001). This mechanism has been observed against fungal species such as *Cladosporium cucumerinum*, *B. cinerea*, and *Fusarium oxysporum* f.sp. *radices-lycopersici* (Hajlaou *et al.*, 1994).

In this study, GC-MS analysis was conducted on the most effective algal extracts against the tested plant-pathogenic fungi. The extracts from *Ulva* sp. (UE and UM) and *Gracilaria* sp. (GE) were subjected to GC-MS analysis, which revealed a variety of diverse compounds. *Ulva* extracts displayed a total of three peaks for ethyl acetate and nine peaks for methanolic extracts. The ethyl acetate extract of *Gracilaria* exhibited seven peaks. To identify their bioactivity, the compounds were compared with previously isolated substances and predicted using the PASS WAY2DRUG online server. It predicts a compound's activity spectrum as probable activity (Pa) and probable inactivity (Pi), with values ranging from 0.000 to 1.000. A compound is considered experimentally active if Pa>Pi (Chy *et al.*, 2019).

Results indicated that three phytocompounds were identified in the UE extract, with Dihydroactinidiolide (retention time RT= 14.94 min) and 8-Heptadecene (RT=16.94 min) being the main chemical constituents with potential antifungal activity, as shown in Table 2. For the methanolic extract of *Ulva*, nine compounds were identified. Among these, 17-Octadecenal (RT=2.33 min), Palmitic acid (RT= 16.80 min), 1,2-Benzisothiazol-3-amine (RT= 25.77 min), and Phenylephrine (RT=01.10 min) have antifungal potential according to PASS predictions and previous studies (Abbassy *et al.*, 2014; Shobier *et al.*, 2016). Interestingly, this is the first report of the compound 17-Octadecenal (RT=2.33 min) in the UM extract with potential antifungal activity.

Previous phytochemical investigations have identified different chemical compounds, including those reported in this study, in various extracts of *Ulva* sp. For instance, an ethyl acetate extract of *Ulva* collected from the Alexandria coast, Egypt found to contain Dichloroacetic acid, heptadecyl ester, (9Z)-9,17-Octadecadienal, and 8-Heptadecene (Shobier *et al.*, 2016). Also, Johnson *et al*. (2014) found that an ethanolic extract of *U. lactuca* from the south coast of India contains seventeen different chemical constituents, including 7-Hexadecene, 8-Heptadecene, Hexadecanoic acid, and 6,9,12,15-octadecatetraenoate. Moreover, Abbassy *et al*. (2014) reported that *Ulva*-methanol extract contains 42 components, with the main five being 1,2-benzene dicarboxylic acid, bis(2-ethylhexyl) ester, palmitic acid, benzene,1,2,4-trimethyl, 8-octadecanoic acid methyl ester and benzene,1-ethyl-2-methyl.

Seven phytocompounds were identified in the GE extract. Among these Dihydroactinidiolide (RT= 25.68 min), (E)-5-Octadecene (RT= 07.00 min), Heptadecane (RT=14.94 min) and 6,10,14-Trimethylpentadecan-2-one (RT= 16.00 min) are notable constituents with potential antifungal activity according to PASS predictions. Ragunathan *et al*. (2019) identified n-Hexadecanoic acid, Heptadecane, Pentadecanoic acid, Oleic acid, and N-(5-chloro-2-hydroxyphenyl)dodecanamide as the most abundant compounds in GE extracts. This suggests that the antifungal activity of these extracts may result from the collective effect of multiple compounds, rather than a single component.

5. CONCLUSION

GC-MS analysis revealed the presence of potential antifungal compounds in UE extract which exhibited the highest inhibition against *Diaporthe eugeniae* and *Pseudopestalotiopsis theae*, and UM extract which had the highest antifungal activity against *Lasiodiplodia theobromae.* Furthermore, these extracts have demonstrated antifungal potential, indicating their possible future applications in sustainable agriculture and the development of novel fungicides to protect crops from fungal pathogens.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Conceptualization, supervision, fund acquisition, reviewing, and editing were done by HMH, PE, and RPW. BKDMR, AHDA, and BMCMB contributed to designing the study, material preparation, data collection, and data analysis. The initial draft of the manuscript was written by BKDMR, AHDA, and BMCMB, with input from all authors on earlier versions. All authors read and approved the final version of the manuscript.

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Research Article

Investigation of the antimicrobial and antibiofilm effect of plant *Consolida orientalis* on methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus sp.* (VRE)

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Abstract: This study was planned to investigate the antimicrobial and antibiofilm effects of *Consolida orientalis* on MRSA and VRE. MRSA and VRE strains isolated from patients admitted to Sivas Cumhuriyet University Medical Faculty Practice and Research Hospital were used in the study. The antimicrobial activity of *C. orientalis* was investigated by microdilution broth method, biofilm formation activity of microorganisms by spectrophotometric plate method and antibiofilm activity of plant extract by microtiter plate method. According to the results, MRSA strains had Minimum Inhibitory Concentration (MIC) values between 0.15 and >5 mg/mL while VRE strains had MIC values between 0.625 and 2.5 mg/mL. Twenty MRSA strains were observed to form biofilm at various levels, 8 of which were strong, 10 were moderate and 2 were weak. Sixteen strains formed biofilms, 1 of which was strong, 15 of which was weak, and 4 strains did not form biofilms. In conclusion, *C. orientalis* plant extract showed moderate to weak antimicrobial activity against MRSA and VRE pathogens. The presence of the substance 2-ethylacridine, which is hypothesised to possess anti-biofilm properties, was identified in the plant extract through the utilisation of gas chromatography/mass spectrometry (GC/MS) analysis. The extract was also found to inhibit biofilm and eradicate bacteria at various levels.

1. INTRODUCTION

Throughout human history, plants and herbal products have been traditionally used to treat a wide range of diseases. The healing power of these herbs has been passed down from generation to generation as a unique heritage. It has been demonstrated that plants possess antimicrobial activity against bacteria, fungi, and viruses. This activity is dependent upon the chemical type, concentration, and properties of the constituents present in the plant. Therefore, the use of herbal medicines to treat microorganism infections shows promise. These studies aim to clarify the chemical components and mechanisms of action of these plants, to use them effectively (Erdoğan & Everest, 2013). *Staphylococcus aureus* is a significant contributor to community and nosocomial infections. It is responsible for a range of skin and soft tissue infections, including impetigo, folliculitis, carbuncle, furuncle, cellulitis, as well as common systemic infections such as bacteremia, endocarditis, meningitis, pericarditis, pneumonia, osteomyelitis

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and septic arthritis. It also causes a number of toxigenic syndromes, including toxic shock syndrome, septic shock, scalded skin syndrome, and food poisoning (Tong *et al*., 2015).

Clinically, the high level of resistance of *S. aureus* to several classes of antibiotics is a significant problem that limits treatment options. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) occurred within two years of the introduction of penicillin, with the first strain identified in 1942. In 1960, the MRSA strain was first identified following the introduction of methicillin, which was developed in the late 1950s, into clinical use. Epidemics linked to *S. aureus* resistance to various antibiotics have emerged in waves. Infections caused by penicillin-resistant *S. aureus* strains were initially largely confined to Europe. However, new strains have been emerging since the 1980s, causing catastrophic worldwide outbreaks (Laghundi & Zhang, 2018). Enterococci are a natural flora element that colonises the gastrointestinal tract of humans and animals. Although enterococci are typically present in low numbers in the gastrointestinal tract, an increased density of these bacteria is an important risk factor for nosocomial enterococcal infection. Resistant enterococcal infections may develop in patients who are treated in hospital intensive care units, immunocompromised, have foreign bodies such as catheters, or receive multiple and various antibiotic treatments. It is important to note that although these infections usually show low virulence, they can still pose a significant risk to patients (Arias & Murray, 2012).

Enterococci are significant causative agents of nosocomial infections, in particular affect the urinary tract, soft tissues, and are frequently associated with medical devices. These infections are a global problem, leading to prolonged hospital stays and increased treatment costs. Given that the risk of treatment failure and mortality is increased in infections caused by strains resistant to multiple antimicrobial agents, it is the importance to continue research into the development of effective treatments (García-Solache & Rice, 2019). Biofilm is defined as “an Extracellular Polymeric Matrix (EPM) formed by microorganisms that are irreversibly attached to a surface, interfaces, each other, or a substrate. These microorganisms exhibit different phenotypes depending on different microbial growth physiologies and gene transcription” (Donlan & Costerton, 2002).

From a medical perspective, biofilms have a wide range of effects. They facilitate bacterial attachment and play a very important role in antibiotic resistance. Research indicates that there are variations in antibiotic susceptibility observed between microorganisms present in biofilms and those in their planktonic counterparts. Furthermore, biofilms can cause inflammation by stimulating the host's immune response against infected biomedical implants. This demonstrates that biofilm formation in medical infections can have significant impacts, ranging from increased virulence of the microorganism to resistance to treatment (Öztürk *et al*., 2008).

Phenolic compounds present in plants and herbal products have been demonstrated to exhibit antibiofilm activity, in addition to their antibacterial effects. Some plant extracts have been found to inhibit Quorum Sensing (QS), which facilitate communication between microorganisms (Truchado *et al*., 2015).

The inadequacy of commonly used antimicrobial treatments against biofilms has prompted researchers to identify and develop new natural antimicrobial agents. Plant constituents and essential oils with antimicrobial activity are expected to have significant potential in the fight against biofilms. Many studies have demonstrated that certain plants, including *Rosmarinus officinalis*, *Juglans regia*, *Rosa canina*, *Castanea sativa*, and *Malva sylvestris*, have an antibiofilm effect on MRSA (Quave *et al*., 2008). Furthermore, *Mentha piperita* has been demonstrated to possess an antibiofilm effect on *Pseudomonas aeruginosa* and *Candida albicans* (Sandasi *et al*., 2011), *Zingiber officinale* on *P. aeruginosa* (Yahya *et al*., 2013), and *Origanum vulgare* has been shown to have an antibiofilm effect on *S. aureus* and *S. epidermidis* (Nostro *et al*., 2007).

The *Consolida* species, which have important medicinal value besides being ornamental plants, are employed in the treatment of a range of ailments, including traumatic injury, rheumatism,

sciatica and enteritis, in various countries, including Turkey, China and others, as well as in some regions, particularly in the Mediterranean and Western Asia. The isolation of compounds and plant extracts from *Consolida* plants has revealed a range of biological activities, including antiparasitic, antifungal, antiviral, anticancer, antioxidant and insecticidal effects. Some components of these plants have been identified as possessing significant potential for exploitation in the development of novel applications, including antitumor and antioxidant activities (Yin, Cai & Ding, 2020). In the light of this information, it is thought that it may be useful to evaluate the antimicrobial and antibiofilm activity of *C. orientalis* plant which grows spontaneously in our province.

The aim of this study was to identify the biofilm-forming properties of MRSA and VRE strains isolated from hospitalized patients and to determine the antimicrobial and antibiofilm effects of *Consolida orientalis* extract on these microorganisms.

2. MATERIAL and METHODS

2.1. Collection and Typing of Plant Samples

In June 2019, *C. orientalis* (Gay) Schröd. plants were collected from an area located at 39° 42' 11" N, 37° 0' 56" E, at an altitude of 1250 m in Sivas province, Central district. The identification of the plant specimens was conducted by Asst. Prof. Dr. Hülya Özpınar of the Faculty of Pharmacy at Sivas Cumhuriyet University, Department of Pharmaceutical Botany.

2.2. Obtaining the Plant Extract

In the present study, the aerial parts of *C. orientalis* (including flowers and seeds) were utilized. The collected plants were cleaned by use of tap water and distilled water, after which they were dried on blotting paper. Thereafter 300 mL of ethanol were added to 100 g of the grounded plant sample, which was then shaken at 150 RPM for 24 hours at room temperature. Subsequently, the mixture was filtered and the ethanol was removed by rotary evaporator (Buchi R-100 equipped with Vacuum Pump V-300 and Control unit I-300) (Özpınar, 2020).

2.3. Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The chemical constituents of *C. orientalis* plant extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) at the Giresun University Central Research Laboratory Application and Research Center (GRUMLAB). An Agilent model 7890A (5975C inert MSD) instrument and HP5MS type column were used for the study.

2.4. Microorganisms Used in the Study

In this study the used strains of MRSA and VRE were isolated from patients admitted to Sivas Cumhuriyet University Medical Faculty Application and Research Hospital. A total of 40 strains, 20 from each microorganism group, were isolated. The microorganisms were identified using the Microflex LT MALDI-TOF MS (Bruker Daltonics, Germany) and antimicrobial susceptibility tests were conducted on the identified strains using the Phoenix 100® system (Becton Dickinson, USA). The bacterial isolates were stored in a deep freezer at -20 ºC. The isolates were passaged on blood agar medium and then incubated overnight at 37 ºC.

2.5. Investigation of Antimicrobial Activity of *C. orientalis* Plant Extract

The microdilution broth method was employed to identify the antimicrobial activity of the *C. orientalis* plant extract. The plant extract was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL. A volume of 90 µL Mueller-Hınton Broth (MHB) medium and 10 µL extract was added to the first row of wells, while 50 µL MHB was added to the remaining wells. Serial dilution was performed by transferring 50 µL of the mixture from the first row to the second row of wells. Then 50 µL of a bacterial suspension, adjusted to a turbidity of 0.5 according to McFarland, was added to each well (CLSI, 2012). Wells in the seventh row were used as growth controls and wells in the eighth row were used as sterility controls. The microplates incubated at a temperature of 37 ± 0.1 °C for 24 hours. The extract concentration

in the first well without visible growth was considered the MIC value. The procedure was repeated three times.

The MIC results were considered effective if they were less than 100 µg/mL, moderately effective if they were between 100 and 625 µg/mL, and weakly effective if they exceeded 625 µg/mL (Awouafack *et al*., 2013; Kuete, 2010).

2.6. Investigation of Biofilm Formation in Microtiter Plates

The biofilm formation activity of microorganisms was quantified using the spectrophotometric plate method (Stepanović *et al*., 2007). After passaging the bacterial isolates on a blood agar medium, they were incubated at 37°C for 24 hours. Then they were suspended in Tryptic Soy Broth (TSB) containing 1% glucose adjusted to 0.5 McFarland turbidity. Subsequently, 200 µL of the bacterial suspensions were transferred to the wells in the microplate. The well containing only 200µL medium was considered the negative control. The microtiter plates were incubated at 37°C for 24 hours, after which the wells were gently emptied and washed three times with phosphate-buffered saline (PBS). After drying at room temperature, 200 µL of 0.1% crystal violet stain was added to wells and kept for 15 min. The absorbance values of the microplates were recorded at a wavelength of 570 nm using the SPECTROstar® Nano spectrophotometer (BMG Labtech, USA).

The formation of biofilms was evaluated according to the scale in Table 1 based on the absorbance value of the negative control (Chusri *et al*., 2012). The study was conducted in triplicate for each strain, with the amount of biofilm formed calculated by averaging the optical density values of each replicate.

Table 1. Biofilm formation scale.

OD ≤ ODc	Non-Biofilm Forming	0
ODc < OD ≤ 2 ODc	Weak Biofilm	I
2 ODc < OD ≤ 4 ODc	Moderate Biofilm	II
4 ODc < OD	Strong Biofilm	III

ODc: Optical Density of Negative Control
OD: Optical Density of Bacterial Biofilm

2.7. Investigation of Antibiofilm Effect of Plant Extract

The anti-biofilm activity of the extract obtained from the *C. orientalis* plant was investigated using the microtiter plate method (Celik *et al*., 2015). The study included nine strains that formed strong biofilm structures. Eight MRSA strains and one was a VRE strain.

2.8. Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

The utilisation of the extract at the MIC dose or higher doses will inhibit the growth of the planktonic form of the bacteria or result in bacterial death. Consequently, there will be no microorganisms present in the environment to form biofilms. For this reason, in the present study, a subinhibitory dose MIC/2 was to investigate doses that do not have lethal properties but have antibiofilm activity (Das, 2018).

The study was continued with eight MRSA and one VRE strains that exhibited a strong biofilm-forming ability. Bacterial suspensions were prepared by adjusting to McFarland 0.5 turbidity in TSB medium containing 1% glucose. Following the addition of 100 µL of bacterial suspension to the wells, 100 µL of plant extract was added at a concentration the MIC/2 for each strain. The procedure was applied to three wells for each sample. The three wells that received only 200 µL of bacterial suspension were considered positive controls, while the wells that received only 200 µL of medium were considered negative controls. Following the incubation, the microplates were washed three times with PBS, dried at room temperature, and stained with crystal violet. The absorbance values were then measured at a wavelength of 570 nm. Based on the obtained data, the inhibitory effect of the plant extract on biofilm formation

was calculated as a percentage value. The percentage of inhibition is calculated according to the following formula (Onsare & Arora, 2015).

$$\%\ inhibisyon = \frac{\mathrm{OD}_c - \mathrm{OD}_{MIC/2}}{\mathrm{OD}_c} \times 100$$

OD_c; optical density value of the positive control wells
$OD_{MIC/2}$; optical density of wells treated with extract at submic (MIC/2) concentration value

2.9. Determination of Minimum Biofilm Eradication Concentration (MBEC)

Eight MRSA and one VRE strains were adjusted to McFarland 0.5 turbidity in TSB containing 1% glucose. Following this, 200 µL of bacterial suspension was added to the wells and incubated at 37°C for 48 hours. After the incubation period, the bacterial suspensions were removed from the wells and 200 µL of the extracts prepared at MIC/2 concentration were added to all wells except the positive control well. Wells that were added with 200 µL of medium were designated as the negative control. The microplates were incubated at 37°C for 24 hours, after which the wells were emptied and washed with PBS. Subsequently, the samples were dried and stained, after which the absorbance values were measured. The data were then compared with the positive control, and the percentage eradication value was calculated (Onsare & Arora, 2015).

3. RESULTS

3.1. GC/MS Analysis Results of *C. orientalis* Extract

The analysis revealed that the plant extract contained 2-Ethylacridine. Furthermore, this substance was identified in the GC/MS analysis of the *B. firmus* fraction, which demonstrated antibiofilm activity in the study entitled "Antibiofilm activity of symbiotic *Bacillus* species associated with marine gastropods" by Viju *et al*. (2020). The results of the GC/MS analysis of the *C. orientalis* plant are given in the table (Table 2).

Table 2. GC/MS results of *C. orientalis* plant extract

Componend name	RT	Area (%)
Octanal	9.620	0.07
Nonanal	13.254	0.10
1-Dodecene	16.355	0.09
Octanoic acid	16.612	0.15
1-Hexadecene	22.632	0.12
3-Octadecene	22.632	0.12
Nonanoic acid	25.710	0.21
Tetradecanoic acid	32.863	0.12
2-Pentadecanone	33.956	0.48
n-Hexadecanoic acid	36.387	0.17
9-Octadecenamide	40.399	0.27
Octadecanoic acid	40.627	1.27
Linoleic acid	40.891	0.24
Eicosadienoic acid	40.999	0.10
Tetracosenoic acid	43.340	0.45
Oleic Acid	46.504	0.87
Cis-Dihidrocarvone	46.716	0.18
Eicosane	49.016	0.21
2-Ethylacridine	53.296	0.20

RT; Retention Time
Area ; % area value per analyte

3.2. Microdilution Broth Method Results

The plant extract derived from the aerial parts of *C. orientalis* was subjected to testing 40 bacterial strains, including 20 MRSA and 20 VRE, in order to ascertain its efficacy. The results

demonstrated variability dependent on the bacterial isolates. The MIC values are summarized in Table 3.

Table 3. MIC results of *C. orientalis* plant extract (mg/mL).

Sample No.	MIC (mg/mL)		Sample No.	MIC (mg/mL)	
	MRSA	VRE		MRSA	VRE
1	0.31	1.25	11	>5	1.25
2	0.31	1.25	12	0.15	1.25
3	1.25	1.25	13	0.625	2.5
4	0.31	1.25	14	1.25	1.25
5	2.5	1.25	15	0.625	1.25
6	0.31	2.5	16	0.625	2.5
7	0.15	0.625	17	1.25	2.5
8	1.25	1.25	18	0.31	1.25
9	1.25	1.25	19	1.25	0.625
10	0.31	2.5	20	0.625	0.625

3.3. Biofilm Formation Activity Results

The study examined the biofilm formation potential of resistant bacteria that caused treatment problems. The majority of these bacteria were found to form biofilms (Table 4).

Table 4. Biofilm forming rates of microorganisms.

Microorganisms (s)	Biofilm				
	Creator				Non-Creator
	Strong	Moderate	Weak	Total s (%)	s (%)
MRSA (20)	8	10	2	20 (100)	-
VRE (20)	1	-	15	16 (80)	4 (20)
Total (40)	9	10	18	36 (90)	4(10)

s: Number

3.4. Antibiofilm Activity Results

In order to determine the in vitro antibiofilm effect of the plant extract, the study continued with samples numbered 5, 6, 9, 10, 12, 14, 17, and 20 from the MRSA group, which had strong biofilm-forming properties, and sample number 14 from the VRE group. The MIC/2 values of these strains have previously been determined and applied to bacteria in the MBIC and MBEC stages.

3.5. MBIC Results

The results demonstrate that the plant extract inhibited biofilm formation in MRSA (5,10,17,20) and VRE (14) samples to varying degrees. The percentages of inhibition were calculated (Onsare & Arora, 2015) from the optical density values obtained and are presented in Table 5.

Table 5. Inhibition at subMIC (MIC/2) concentration (%).

Microorganisms	MIC/2 (mg/mL)	% MBIC
MRSA-05	1.25	26±8.3
MRSA-06	0.15	-*
MRSA-09	0.625	-*
MRSA-10	0.15	85±0.8
MRSA-12	0.07	-*
MRSA-14	0.625	-*
MRSA-17	0.625	35±13.3
MRSA-20	0.31	53±16.4
VRE-14	0.625	12±1.6

ubMIC (MIC/2) concentration (%).
* Inhibition not observed

3.6. MBEC Results

The results of the MBEC study demonstrate that biofilms were eradicated to varying degrees in samples 5, 6, 9, 14, 17, and 20 from the MRSA group and sample 14 from the VRE group. The percentage of eradication is presented in Table 6.

Table 6. Eradication at subMIC (MIC/2) concentration (%).

Microorganisms	MIC/2 (mg/mL)	% MBEC
MRSA-05	1.25	61±1.6
MRSA-06	0.15	19±9.4
MRSA-09	0.625	49±5.2
MRSA-10	0.15	-*
MRSA-12	0.07	-*
MRSA-14	0.625	63±2.4
MRSA-17	0.625	72±1.5
MRSA-20	0.31	13±9.5
VRE-14	0.625	20±6.4

*Eradication not observed

4. DISCUSSION and CONCLUSION

Antimicrobial resistance in microorganisms is a global issue that results in high mortality rates worldwide, regardless of a country's development status. Microorganisms are capable of developing resistance to antimicrobial compounds through a process of mutation and natural gene transfer. The intrinsic antibiotic resistance in microorganisms is typically associated with the cellular impermeability of the microorganisms to antimicrobial agents. Moreover, Nadaf and colleagues observed an increase in the expression of drug resistance genes in bacterial strains that were clustered together (Nadaf *et al*., 2018).

Although synthetic drug research is emphasized as a means of combating emerging resistance, the potential toxicity and side effects of many synthetic drugs have increased interest on medicinal plants. This has prompted microbiologists globally to devise innovative antimicrobial agents and assess the potential of natural plant-derived substances as alternatives to chemical antimicrobials (Maregesi *et al*., 2008). It is a widely acknowledged fact that plant phytochemicals exhibit antibacterial activity against free bacterial cells and have the capacity to reduce biofilm development through specific mechanisms (Nadaf *et al.*, 2018).

It is often observed that compounds with medicinal and antimicrobial properties derived from plants show potential activity against biofilm formation. There has been considerable interest among researchers in extracts and essential oils derived from medicinal plants, which have been subject of extensive study. Additionally, plant extracts are commonly used in the pharmaceutical industry due to their bioactive compounds with antimicrobial properties. Many studies have demonstrated that solvent extracts and plant fractions possess biofilm-inhibitory effects against various bacteria and fungi. The antimicrobial and bactericidal properties of essential oils have been demonstrated to disrupt the environmental conditions required for the growth of many bacteria and fungi (Bazargani & Rohloff, 2016). A substantial body of research exists on the antimicrobial and antibiofilm properties of essential oils.

The present study investigated the efficacy of ethanolic extract derived from the *C. orientalis* plant on 40 diverse bacterial strains at concentrations spanning from 5 to 0.15 mg/mL. The results demonstrated that the response of the bacterial isolates differed. A few studies have investigated the antimicrobial effect of *C. orientalis* and other *Consolida* species. Our results are consistent with these findings (Rahdari *et al*., 2010; Rochetti, 2020). Moreover, a considerable body of research has been conducted in the academic literature on the antimicrobial activity of extracts derived from diverse plant parts, including leaves, flowers, and above-ground portions. These studies have employed a range of solvents to investigate the

antimicrobial potential of these extracts (Avşar *et al*., 2016; Yetgin *et al*., 2017; İlkimen & Gülbandılar, 2018).

In this study, all 20 MRSA strains were observed to form biofilms in varying degrees. Eight of the strains exhibited strong biofilm formation, 10 exhibited moderate biofilm formation, and 2 exhibited weak biofilm formation. It was observed that 16 VRE strains, 1 of which was strong and 15 of which were weak, formed biofilm, while 4 VRE strains did not form biofilm. The biofilm-forming properties of the MRSA group in our study were found to be consistent with the findings of İştar (2018). In their study on "Defining conditions for biofilm inhibition and eradication tests for Gram-positive clinical reference strains", observed that enterococci formed an optimal biofilm with an extended incubation period in TSB supplemented with 1% glucose (Cruz *et al*., 2018). The lower biofilm formation rates observed in the VRE group in our study may have been because the same incubation time was applied to both the MRSA and VRE groups.

The study applied the extract obtained from the *C. orientalis* plant to bacteria and observed biofilm inhibition at various levels between 12% and 85% in a total of five samples. Four samples were from the MRSA group and one from the VRE group. Furthermore, biofilm eradication was observed to occur between 13% and 72% in seven samples. Six of the samples were from the MRSA group, and one of the samples was from the VRE group. A search of the literature revealed no studies investigating the antibiofilm activity of *C. orientalis* plant extract. Nevertheless, a number of studies have documented the anti-biofilm activity of diverse natural compounds, including those of Arslan (2019), Atalan (2019), Balaban (2018), Erdönmez *et al*. (2018), Famuyide *et al*. (2019), Göse (2019), Karaca *et al.* (2017), Nadaf *et al.* (2018), and Tozyılmaz (2019).

In 2020, Viju *et al*. conducted a study on three *Bacillus* species, *B. firmus, B. cereus*, and *B. subtilis*, which live symbiotically with gastropods. The researchers prepared extracts from the strains and investigated their activity on the biofilm-forming marine bacteria *Alteromonas sp*. The results demonstrated that the symbiotic bacterial extracts exhibited strong inhibitory effects on biofilm formation, with *B. cereus, B. subtilis*, and *B. firmus* exhibiting the highest inhibition, respectively. The GC-MS analysis of the fraction of *B. firmus* exhibiting antibiofilm activity revealed the presence of a variety of compounds. These included 2-ethylacridine, indolizin, and anthranilic acid.

In certain studies, researchers employed extracts and essential oils derived from diverse plant species, including various plant parts. The studies reported varying levels of antimicrobial, antifungal, and antibiofilm activity on different microorganisms (Nostro *et al.*, 2007; Adukwu *et al*., 2012; Taweechaisupapong *et al*., 2012; Selim *et al*., 2014; Çelik *et al*., 2015; Bazargani & Rohloff, 2016; Haiyan *et al*., 2016; Merghni *et al.*, 2016; Tutar, 2018).

Honeybees are capable of producing a variety of products, including honey, propolis, bee venom, pollen, beeswax, and royal jelly. Such products possess beneficial biological properties and are applicable in many fields, making them popular alternative products in medicine due to their chemical structure. According to ancient Greek writings, propolis was used to treat festering wounds and dental caries. During the Roman period, propolis was incorporated into a poultice-like mixture applied to wounds (Alıç, 2015). In light of these findings, a considerable body of research has been done to evaluate the anti-biofilm efficacy of honey.

In a study conducted by Kim *et al*. (2019), it was observed that the bacteriocin produced by *Lactobacillus brevis* DF01 was effective in reducing biofilm formation. The findings of the study indicate that DF01 bacteriocin affects the formation of biofilms, yet does not disrupt established biofilms.

Biofilm-producing bacteria are frequently associated with the pathogenesis of chronic disease processes, which may give rise to the persistence of localized inflammation and the subsequent damage to surrounding tissues. In certain cases, the complications that result from such

infections can prove to be potentially life-threatening. It has been demonstrated that the opportunistic pathogens most frequently associated with the formation of biofilms are *S. aureus* and *Candida albicans* (Nadaf *et al*., 2018). The antibiotics currently in use are inadequate in treating biofilm-associated infections due to their high MIC and MBC values, which can be toxic to the body. The development of anti-biofilm molecules that are effective in the reduction and elimination of biofilm-related infections is of critical importance (Roy *et al*., 2018). A considerable number of compounds derived from natural sources, including plants, animals and microbes, have been identified and documented as exhibiting antibiofilm activity. The aforementioned compounds are obtained from renewable resources and can be employed as antibiofilm coatings (Viju, Punitha, & Satheesh, 2020).

In light of current knowledge, the importance of obtaining chemical compounds with high antimicrobial and antibiofilm activity as well as low toxicity from natural sources is increasing. The method in question represents a promising approach to combating microbial infections, particularly given the mounting challenge of drug resistance and the biofilm-forming capabilities of microorganisms, which increase pathogenicity, placing an additional burden on therapeutic intervention. In light of the mounting posed by biofilm infections, each study on this subject contributes invaluable data to the existing body of literature, representing a significant advancement in the field. Further studies are recommended to investigate the effects of the substances present in the chemical composition of *C. orientalis*, which is known for its showy purple leaves in the fields in spring.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Ethical principles were complied with at every stage of the research, and written permission was obtained from Sivas Cumhuriyet University Non-Interventional Clinical Research Ethics Committee with the decision dated 07.08.2019 and numbered 2019-08/10 before starting the applications.

Authorship Contribution Statement

Gonca Şimşek: Investigation, Resources, Visualization, Formal Analysis, and Writing. **Ömer Poyraz**: Methodology, Supervision, and Validation.

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Research Article

Profiles of volatile metabolite compounds of lotus tempeh: In-RSM-Boxbehken approach

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Abstract: The newest fermented product that uses lotus seeds is tempeh. Processing conditions that vary in generating tempeh products are considered in determining quantity and quality. This research aimed to optimize processing conditions (yeast ratio, soaking, and fermentation time) on lotus tempeh. In this research, the program design used RSM Box-Behnken Design to investigate and select the best combination process for lotus tempeh making. The response of protein and tannin was seen in the range protein value was 5.60% up to 9.80%, while the tannin content was 14.71 to 21.59 %w/w GAE. It was established that the highest protein content was achieved in a 1 g starter, 15 h soaking time, and 36 h fermentation time, whereas the condition of 0.75 g starter, 15 h soaking time, and 48 h fermentation time led to the highest tannin content. The ideal conditions for the production of lotus tempeh have been identified as those that yield a total of 17 amino acids and 46 volatile compounds. Indeed, the findings of this study have indicated that the fabrication of lotus tempeh is most efficiently achieved under specific conditions. Furthermore, the research has suggested that other components hold significant potential as protein and tannin sources, warranting further investigation.

1. INTRODUCTION

The seed of *Nelumbo nucifera* Gaertn, an amphibian plant in the *Nelumbonaceae* that is usually cultivated and eaten in China, is known as Lotus seed or Fragrant pink/white blossoms with expansive and round leaves (Bangar *et al*., 2022; Nainggolan *et al*., 2022). The organisms under consideration inhabit a milieu characterized by oceanic verdure, wherein stagnant or lethargic freshwater conditions prevail. In reality, the lotus can adjust to different sea-going natural surroundings, going from shallow lakes to far-reaching lakes (Gowthami *et al*., 2021). The diet of the subjects had a significant impact on their nutrient levels. A range of nutrients, including dietary fiber, carbohydrates, amino acids, phenols and other trace components, have been identified in Lotus seeds (Nainggolan *et al*., 2022). One of the newest fermented products that used lotus seeds was natto and tempeh. Tempeh is a traditional food which commonly made from soybeans through a fermentation process.

The fermentation process has been demonstrated to enhance the phytochemical content of foodstuffs, including free isoflavones, complex proteins and carbohydrates, and to produce

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probiotics that may prove beneficial to the gastrointestinal tract (Dhull *et al*., 2022; Salman *et al*., 2022). A plethora of studies have been conducted on the health-promoting potential of lotus, encompassing antioxidant, cytotoxic, and immunostimulatory properties (Bangar *et al*., 2022). Ridhowati *et al.* (2022) determined that the isoflavones contained in lotus tempeh have potential as anticancer breasts. According to Reddy *et al.* (2022), there is an overhaul of not only protein, fat, and carbohydrate but also bioactive compounds during tempeh processing. A previous study concluded the stage and time of processing to make lotus tempeh could affect the lotus tempeh quality (Ridhowati *et al*., 2022).

The processing conditions which vary in their capacity to generate tempeh fermentation must be given full consideration when determining quantity and quality. In this research, the program design used RSM Box-Behnken Design for investigating and selecting the best combination process for lotus tempeh making. The advantages of this program can be used for analyzing and modeling a problem with one or more treatments in research (Montgomery, 2012; Ridhowati *et al*., 2022). This method not only defines the effect of the independent variable but also produces a mathematical model, which describes a chemical or biochemical process. In addition, the method has the advantage that it does not require a large number of trials or a protracted period of time (Montgomery, 2012). This research aimed to optimize processing conditions (yeast ratio, soaking, and fermentation time) on tempeh processing by using Response Surface Methodology (RSM)-Box Behnken. This research is expected to be basic information in the process scale-up for lotus tempeh processing with optimized protein, tannin, and volatile profile compounds.

2. MATERIAL and METHODS

2.1. Preparation Lotus Tempeh

The making of lotus tempeh processing was determined by the methods of Ridhowati *et al.* (2022). Lotus seeds (250 g in 2 L of aquadest) were subjected to boiling for 15 minutes, following which they were soaked in soy acid (pH 4-5). The duration of this soaking process was customized according to the parameters outlined in Table 1. The loose seed hulls were removed. The dehulled seeds were washed and weighed with the sample proportion (w/w). The dehulled beans were boiled using aquadest (2 L) for 15 min. All of the formula samples were inoculated exploitation flora spores (*Rhizopus sp*.) to the cooled beans employing a quantitative relation (w/w) in Table 1, and sporulated beans were later on placed in perforated plastic baggage (13 cm x 20 cm) wherein its thickness was 1.5 cm for fungal development. The samples were subjected to the designated formulation during the incubation process (Table 1).

2.2. Proximate Analysis

The proximate and amino acid analyses were conducted according to Horwitz and Latimer (2005).

2.2.1. *Moisture content*

A total of 5 g of sample was weighed quickly into a dry test cup, then homogenized, and dried in an oven thermogravimetry (Memmert UN30) at 105^{o}C for 6 h. Then, it was cooled in a desiccator until it was balanced. The sample was put back in the oven until a constant weight was obtained. The moisture content was calculated using the following formula:

$$\text{Moisture content (\%)} = \frac{b-(c-a)}{b} x100\%$$

where

a = the constant weight of the dry test cup (g)
b = the sample weight (g)
c = the constant weight of dry test cup and dry sample (g)

2.2.2. *Ash content*

A total of 5g samples were weighed and subsequently placed in the furnace (SNOL Merck, SNOL 3/1100) at a temperature of 300°C. The temperature was then increased to 550°C at a rate appropriate to the material characteristics (generally 5-7 hours) until the oxidation of all carbon had been completed. The sample was subjected to a process of evaporation, continuing until the desired consistency of dryness was achieved (a grayish-white appearance). Next, the furnace was turned off and can be opened after the temperature reaches 250^{o}C or less. The ash content in the sample can be calculated using the formula:

$$\text{Ash content (\%)} = \frac{W2-W0}{W1-W0} x100\%$$

where

W_2 = the porcelain cup + sample after ashing (g)
W_0 = the porcelain cup (g)
W_2 = the porcelain cup + sample before ashing (g)

2.2.3. *Fat content*

A total of 5 g of the sample was wrapped in paper Strain, and then covered with cotton wool that had been deprived of fat. The filter paper containing the sample was inserted into Soxhlet extraction kits, and a condenser was installed above it, and the flask fat underneath. A quantity of 80 milliliters of hexane (purchased from Sigma-Aldrich) was added to the flask containing the fat. Subsequently, a minimum of five hours of refluxing was undertaken until the solvent receded into the fat flask and a clear color was achieved. The solvent in the fat flask was then distilled and stored. Then the fat flask containing the extraction results was heated in an oven (Memmert UN30) at 105^{o}C, to evaporate the remaining solvent. Then, it was cooled and weighed until a constant weight was obtained. The calculation of the percentage of fat in the sample can be performed using the following formula:

$$\text{Fat content (\%)} = \frac{Wc-Wa}{Wb} x100$$

where

Wc = the fat flask + fat after extraction (g)
Wa = the fat flask (g)
Wb = Sample (g)

2.2.4. *Protein content*

Protein content was analyzed using Micro-Kjeldahl; 0.5 g samples were added 1.2 g mixed catalyst (Kjeldahl powder, Merck) and 10 mL H_2SO4 (Merck), then boiled in Kjeldahl flask until the solution colored greenish and clear. The solution was subjected to a ten-fold dilution process, whereby 5 mL of the diluted sample was introduced into a distilled flask containing 30% NaOH (Merck) and 1 mL of phenolphthalein (Merck). The neutralization process was conducted for 10 minutes, after which the ammonia gas was collected in a flask containing 2% boric acid (Merck). The nitrogen content of the protein samples was then estimated using titration with 0.01 N HCl (Merck), calculated using the following formula:

$$\text{Protein content (\%)} = \frac{(v1-v2)x\ N\ x\ 14.007\ x\ 6.25}{W} x\ 100\%$$

Where, V_1 and V_2 = titration volume of sample and blank; 14.007 gram is molecular weight (MW) of Nitrogen; N is normality of HCl; and W is the weight of the sample; 6.25 is protein factor for food.

2.2.5. *Carbohydrates by differences*

Carbohydrate content was calculated by the formula
Carbohydrate (%) = 100 % – (protein + fat + moisture + ash)

2.2.6. *Amino Acids analysis*

Fifty milligrams of samples were added to 10 mL HCl 6 M and incubated for 24 h at 100^{o}C. The aliquot of the sample was filtered and the extract was diluted 200 times with milli-Q water. A 300 mL extract was dried and derivatized with 300 mL of 9-fluorenyl methyl-chloroformate (FMOC). A 20 mL aliquot was analyzed using an analytical scale (4.6 x 250 mm^2) SGE Hypersil ODS C18 column (SGE, Dandenong, Australia) kept at 38^{o}C and connected to an HPLC system (GBC, Dandenong, Australia) equipped with a fluorescence detector LC 5100. The mobile phases used were as follows: A: 30 mM ammonium phosphate (pH 6.5) in 15: 85 (v/v) methanol/water; B: 15: 85 (v/v) methanol/water; and C: 90: 10 (v/v) acetonitrile/water. The flow rate was 1.2 mL/min and the gradient, wavelength for Fluorescence detection was 270 and 316 nm. A calibration curve was constructed using a mixture of standard amino acids.

2.3. Total Tannin

The tannin contents were measured as Gallic Acid Equivalents (GAE) based on the method of Ahnan (2020); Horwitz and Latimer (2005). Tannin content analysis was carried out using the Folin-ciocalteu method. The principle of the Folin-Ciocalteu method, namely the oxidation of phenolic compounds by the Folin-Ciocalteu reagent produces a blue solution. Standard solutions were prepared at concentrations ranging from 300 to 800 parts per million (ppm) by combining 1 milligram (mg) of gallic acid with 5 milliliters (ml) of methanol, followed by vortexing. The sample solution was ready. The test was made according to the dilution, 1 ml samples were added with distilled water, 1.5 ml 35% Na_2CO_3, and 0.5 ml of 50% Folin-Ciocalteu reagent up to 5 mL dilution, vortexed, and incubated for 30 min in a closed room, and then, the absorbance was measured using a UV-VIS spectrophotometer Wavelength 760 nm.

2.4. The Volatile Compound

The samples were analyzed for their volatile compounds not only lotus tempeh but also commercial tempeh, based on Kustyawati (2017). Then, they were extracted using a headspace solid phase microextraction (SPME)-GC (Gas Chromatography)- MS (Mass Spectrophotometry) method. 50 grams of sample was put into an Erlenmeyer flask, sealed with aluminum foil with a protective seal, and fitted with a space solid phase microextraction gas-tight syringe. The flask was placed in a water bath at 50^{o}C, wherein the samples would release their volatiles and be absorbed onto SPME for 30 minutes at 50^{o}C. The HP 5890A gas chromatograph associated with an HP 5970 mass-specific indicator (Hewlett Packard) was used to dissect the unpredictable examples. GC-MS was worked at 70 eV in the EI mode over the reach 35-450 amu, section utilized was BP-5x segment (30 mx0.25 mm) with 0.25 µm film thickness to determine the volatiles (Supelco, Sigma-Aldrich Co.). Helium was utilized as a transporter at a stream pace of 1 ml/minute. The volatiles thus collected were subjected to thermal desorption at a temperature of 250°C for two minutes. Thereafter, the temperature of the desorption apparatus was rapidly increased to 60°C and maintained at this level for five minutes. This was followed by a decrease in temperature to 5°C, at which point the process was continued until the temperature had reached 220°C, a process which took ten minutes. The constituents of the tests were likely distinguished by coordinating their mass spectra with those stored in the PC library (NIST98 and Wiley library).

2.5. Experimental Design

The first research started with the quality of material dried lotus seeds including the proximate, amino acid, and tannin analysis. All of the parameter analyses in this research were replicated 3 times. Then, the second stage determined the optimization of tempeh processing; 1) a

formulation design and response, 2) formulation, 3) response analysis, and 4) optimization (Montgomery, 2012; Ridhowati *et al*., 2022). The subsequent stage of the process is verification, which serves to validate the predicted value of the optimum solution response formula (see Table 1).

Table 1. Experimental design of lotus tempeh processing.

Formula	Factor 1	Factor 2	Factor 3
	Total of starter (g)	Soaking time (h)	Fermentation time (h)
1	1	15	36
2	0.5	15	60
3	0.75	15	48
4	0.75	15	48
5	0.75	15	48
6	0.75	24	60
7	0.5	15	36
8	0.75	24	36
9	1	24	48
10	0.5	6	48
11	0.5	24	48
12	1	15	60
13	0.75	6	36
14	1	6	48
15	0.75	6	60

Total of starter (g) : 0.5 (min)- 1 (max)
Soaking time (h) : 6 (min) - 24 (max)
Fermentation time (h) : 36 (min) - 60 (max)

2.6. Statistical Analysis

All stages of the process were analyzed, and each response (protein and tannin contents) was determined for the purpose of optimization in the Minitab 19.0® program. The program is capable of performing optimization procedures according to both variable and measurement data entered into the response.

Once the optimum process conditions were established, the verification steps for lotus tempeh processing were initiated. This process was analyzed in accordance with the formulation that had been optimized through the utilization of the RSM-BoxBehnken design. The verification process was conducted with two repetitions, followed by a comparison with the predicted values of the response variables from the RSM-Box Behnken design. These values were equipped with a prediction of the value of each response, thereby enabling the assessment of the suitability of the verification steps (Ahnan, 2020; Montgomery, 2012).

3. FINDINGS

3.1. Proximate Analysis

The quality substances of raw materials were determined in Table 2. Based on Table 2, the water content of lotus seed as a sample in this study was 12.48%, the value was slightly higher than standardized in Indonesia SNI 3144:2009 and FAO-WHO CODEX STAN 313-2013 for standards tempeh using soybean seed.

Table 2. Results of analysis and quality requirements of dried lotus seeds (% w/w, wet basis).

Parameters	Soybean Tempeh	Lotus Tempeh	Lotus Seeds
Moisture	61.09 ± 0.55	67.23 ± 0.56	12.48 ± 0.09
Ash	0.84 ± 0.04	1.22 ± 0.02	4.36 ± 0.03
Carbohydrates	5.16±1.18	19.54±0.36	71.02 ± 1.64
Protein	17.23±0.86	9.36±0.12	7.67 ± 1.15
Fat	5.05±0.21	5.79±1.88	4.46 ± 0.36

3.2. Optimization of Processing Conditions with RSM-BoxBehnken Design

The total starter, soaking, and fermentation time were selected as optimization conditions for making this product. As demonstrated in Table 3, the range of protein values was from 5.60% to 9.80%, while the tannin content ranged from 14.71% to 21.59% w/w GAE. The highest protein content was observed in the 1 g starter, 15 h soaking time, and 36 h fermentation time condition, while the condition of 0.75 g starter, 15 h soaking time, and 48 h fermentation time yielded the highest tannin content.

Table 3. The protein and tannin contents of lotus tempeh based on process conditions (% w/w, wet basis).

Formulas	Total of starter (g)	Soaking time (h)	Fermentation time (h)	Protein (%w/w)	Tannin (%w/w GAE)
1	1	15	36	9.80 ± 0.01	21.43 ± 0.23
2	0.5	15	60	6.61 ± 0.01	18.94 ± 0.01
3	0.75	15	48	9.36 ±0.00	21.59 ± 0.16
4	0.75	15	48	9.19 ± 0.01	21.45 ± 0.52
5	0.75	15	48	8.75 ± 0.01	20.44 ± 0.54
6	0.75	24	60	5.08 ± 0.00	15.29 ± 0.06
7	0.5	15	36	8.14 ± 0.00	18.69 ± 0.37
8	0.75	24	36	6.30 ± 0.00	16.61 ± 0.04
9	1	24	48	9.19 ±0.01	19.64 ± 0.04
10	0.5	6	48	7.88 ± 0.01	17.42 ± 0.14
11	0.5	24	48	9.14 ± 0.00	19.36 ± 0.04
12	1	15	60	4.68 ± 0.01	14.71 ± 0.25
13	0.75	6	36	5.43 ± 0.00	15.58 ± 0.42
14	1	6	48	7.22 ±0.00	17.49 ± 0.05
15	0.75	6	60	5.60 ± 0.00	15.74 ± 0.05

Regression analysis (Table 4) was plotted to make three-dimensional response surface. Response protein contents would directly be high along with increased soaking time, fermentation time, and interaction between total starter and soaking time which were indicated by a positive constant value (Figure 1A and Table 4).

Table 4. Analysis model for the protein and tannin contents.

Response	Model	Equation	Significant (p<0.05)	Lack of fit (p<0.05)	R^2
Protein	Quadratic Polynomial	-33.6 - 0.3 A +0.578 B + 1.670 C + 8.68 A*A - 0.01438 B*B - 0.01538 C*C + 0.079 A*B - 0.299 A*C - 0.00324 B*C	0.067	0.057	88.05
Tannin	Quadratic Polynomial	- 49.4 +27.5 A +1.197 B +2.204 C - 0.36 A*A - 0.03284 B*B - 0.01872 C*C + 0.023 A*B - 0.581 A*C - 0.00343 B*C	0.031	0.234	91.2

*A = total of starter; B = soaking time; C = fermentation time

The tannin responses had a positive value (Figure 1B and Table 4) for total starter, soaking time, fermentation time, and interaction between total starter and soaking time. All of the responses would decrease if there was correlation with fermentation time that is indicated by negative constant values.

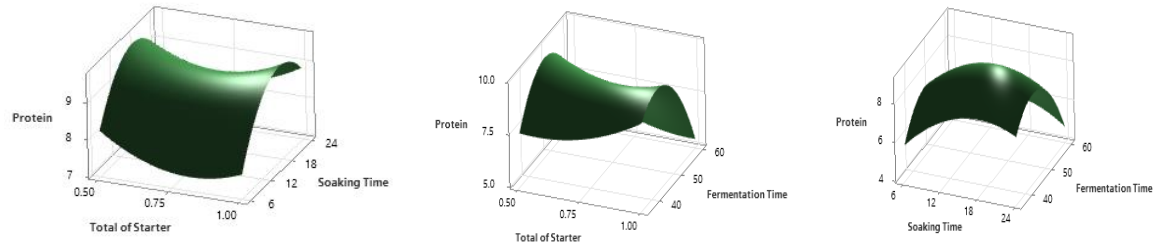

Figure 1A. Response surface plots of protein contents by Total of starter (A), soaking time (B), and fermentation time (C).

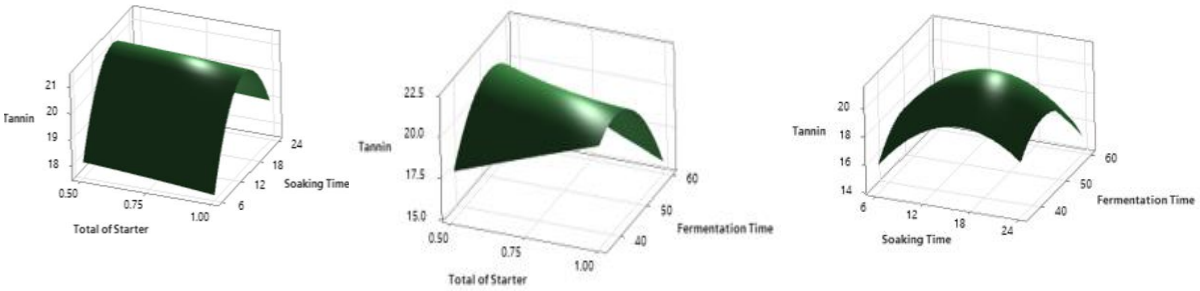

Figure 1B. Response surface plots of tannin contents by Total of starter (A), soaking time (B), and fermentation time (C).

A Box-Behnken response surface methodology (RSM) was employed to optimize the total starter, soaking, and fermentation times. The significance of model parameters was evaluated using analysis of variance (ANOVA) and regression linear (R2) values, and the responses from experimental runs were fitted into a second-order polynomial regression model. Using the desirability principle, the best conditions for protein was in 1 g starter, 15 h soaking time, and 36 h fermentation time, while the condition in 0.75 g starter, 15 h soaking time, and 48 h fermentation time was the tannin contents. The lotus tempeh made in the optimal condition resulted in 17 amino acids and 46 volatile compounds (Table 5). Ridhowati *et al*., (2022) determined that the optimal condition for getting these volatile compounds was 0.5 g starter, 24 h soaking time, and 36 h fermentation time.

Table 5. The profile's chemical lotus tempeh based on the best conditions (% w/w, wet basis).

Samples	Profile's chemical			
	Amino acid (%w/w)		Organic acid (% w/w)*	
Lotus tempeh	Serine	0.44 ± 0.00	Cyclobutanol	2.78
	Glutamate acid	1.05 ± 0.00	Isoamyl-hydride	2.82
	Phenylalanine	0.36 ± 0.00	Acetaldehyde	3.02
	Isoleucine	0.22 ± 0.00	Pentanal	3.71
	Valine	0.26 ± 0.00	Ethyl acetate	4.49
	Alanine	0.35 ± 0.00	Ethene, ethoxy	4.62
	Arginine	0.45 ± 0.00	Ethanol	5.10
	Glycine	0.36 ± 0.00	3-Pentanone	5.62
	Lysine	0.31 ± 0.00	Acetic acid, cyano-	5.98
	Aspartate acid	0.66 ± 0.00	α-Pinene	6.41
	Leucine	0.42 ± 0.00	2-Butanol	6.67
	Tyrosine	0.20 ± 0.00	1-Propanol	6.75
	Proline	0.23 ± 0.00	Dimethylamine	7.16
	Threonine	0.29 ± 0.00	Iso-butanol	7.61
	Histidine	0.16 ± 0.00	3-Pentanol	7.87

Table 5. *Continues.*

	Cystine	0.22 ± 0.00	Isoamyl acetate	8.17
	Methionine	0.01 ± 0.00	β- Myrcene	8.92
			γ-Terpinene	8.98
			α-Ocimene	9.09
			(+)-2-Carene	9.27
			2,3-Epoxybutane	9.41
			Limonene	9.62
			Isoamyl alcohol	9.75
			Furan, 2-pentyl-	10.16
			2-Ethylcyclobutanone	10.37
			Iso-butenyl-carbinol	10.51
			3-Octanone	10.63
			m-Xylene, 5-ethyl-	10.92
			Acetoin	11.19
			Butanal	11.89
			2-Octanol, (R)-	12.15
			2,3-Butanediol	12.23
			1-Hexanol	12.35
			2-Methylcyclohexanol	13.56
			Acetic acid	13.91
			Benzene, 1,2-dichloro-	13.97
			Ethyl 3-hydroxybutyrate	14.11
			2-Anthracenamine	14.47
			Benzaldehyde	15.29
			Iso-butyric acid	15.81
			Caryophyllene	16.49
			Methyl benzoate	16.84
			Naphthalene	18.63
			2-Amino-5-methylbenzoic acid	19.79
			Benzyl alcohol	20.36
			Phenylethyl alcohol	20.88

Note: * (Ridhowati *et al.*, 2022)

4. DISCUSSION and CONCLUSION

A comprehensive understanding of the variation step in tempeh production is instrumental in optimizing the quality of the final product, particularly with regard to proximate substances (see Table 2). In addition, the production step of lotus tempeh was optimized for maximum protein and tannin contents using RSM-Box Behnken with three-level-three factor (Table 3), based on Ahnan (2020) who determined that soaking, incubating, and inoculating were important factors in tempeh making.

It has been demonstrated that an increase in fermentation time is associated with a corresponding increase in protein content (Table 3). There was the activity from the fungi which broke down the protein into many free amino acid compounds during the fermentation process (Bahlawan *et al*., 2022; Erkan *et al*., 2020). On the other hand, fermentation was carried out for 60 hours resulting in decreasing tempeh protein levels, a number of proteins were used as a nitrogen source for growth by *Rhizopus sp*. However, Boutas *et al*. (2022) found no significant change in total crude protein after seven days of germination. This was the result of soaking the seeds for 12 hours and then sprouting them in the light at 20°C. Soaking was commonly processed to produce tempeh ranging from 6 to 24 hours, the lotus seed in this step made it easy

to peel due to hydration mechanism. In the fermentation stage, the temperature was maintained at 25 to 30°C for 36 to 48 hours. This process leads to the proliferation of microorganisms, which in turn stimulates the bioavailability of nutrients and eliminates anti-nutrients. Consequently, this results in the promotion of health, as evidenced by the research conducted by Bahlawan *et al*., (2022), Boutas *et al*., (2022) and Ridhowati *et al*., (2022).

The protein and tannin contents varied depending on each treatment point, which determined the importance of processing conditions for tempeh making. All of the responses had the quadratic polynomial (second-order regression equation) calculated by RSM Box-Behnken for evaluation of the correlation between variables and responses (Table 4). The relationship between the two variables of protein and tannin content was demonstrated in three-dimensional surface plots (three-dimensional responses) based on regression equations (see Figure 1A and 1B). These figures demonstrated that the model fitted the experimental data well and was suitable for optimization.

In the study by Riswanto *et al*. (2021), the impact of varying soybean germination times (28-72 h) on the quality of soymilk was examined. The results indicated that a germination time of 28 h resulted in higher protein and total phenolic content. Concurrent research on soybean bioactive components has indicated that a germination time of 45 hours is the optimal treatment to elevate lunasin and reduce lectin and lipoxygenase activity (Acin-Albiac *et al*., 2021; Nahar *et al*., 2023). However, the extant research is limited in its discussion of the soaking conditions required for bean sprouting, particularly in relation to other parameters. Consequently, there is a necessity to optimize the soaking and germination times to achieve the desired qualitative characteristics of soymilk (He *et al*., 2023; Roasa *et al*., 2021).

The extended germination periods may be a contributing factor to the depletion of nutrients (Feng *et al*., 2022; Roasa *et al*., 2021; Zhang *et al*., 2023). This appears reasonable, given that sprouting has a metabolic process involving the breakdown of nutrients, cotyledon expansion, and energy release (Feng *et al*., 2022; Zhang *et al*., 2023). It has been demonstrated that there is a direct correlation between the total protein content and the duration of the soaking and germination processes (Chai *et al*., 2022; Zhang *et al*., 2023). Sprouting causes the formation of a variety of enzymes, which could be involved in the biosynthesis or degradation of complex proteins. Previous research has found that sprouted soybeans and buckwheat had somewhat higher protein content after 72 hours of germination (Zhang *et al*., 2023)

As Zhang *et al*. (2023) highlighted that the soaking time constituted a pivotal process for products derived from beans. It was determined that an increase in phenolic content, including tannin, was observed in products when compared to their dormant states, with an increase of up to 30% being recorded. Furthermore, the protein content was found to be higher in the sprouted samples than in the non-sprouted ones (Ahnan, 2020; Acin-Albiac *et al*., 2021; Boutas *et al*., 2022). These conditions assisted in breaking the complex compounds until more absorbable forms, hence, enhancing nutritional content, digestibility, and overall functionality (Acin-Albiac *et al*., 2021; Boutas *et al*., 2022). Many factors, including soaking time, germination time, temperature and grain variety can affect the quality of sprouted grain (Chai *et al*., 2022; He *et al*., 2023).

Sprouting is a potent way of increasing the phenolic acids in leguminous seeds, as phenolics are biosynthesized and bioaccumulated in germinating seeds, as a response and defense mechanism of seeds to the adverse environmental stress created by sprouting conditions (Ahnan, 2020; Erkan *et al*., 2020). Sprouting had previously been linked to a decrease in amino acid content (Ahnan, 2020; Feng *et al*., 2022; Ridhowati *et al*., 2022). Hydrolysis, synthesis, and rearrangement of protein moieties were thought to be responsible for the variations in amino acid concentration found in sprouted beans during the first 72 hours of sprouting (Ahnan, 2020; Feng *et al*., 2022; Ridhowati *et al*., 2022). The extraction process in the current study by solid-state fermentation (SSF) of wheat by *Rhizopus oryzae* which helped to release the bound compounds from the matrix (Acin-Albiac *et al*., 2021; Ahnan, 2020).

Sprouting prior to processing into a desired product is a cheap and effective pre-treatment for increasing metabolic activity (Ahnan, 2020; Chai *et al*., 2022). The process of autoclaving and fermentation treatments might alter the diversity and contents of phenolic compounds, resulting in an increased release of free compounds, ferulic acid (Bahlawan *et al*., 2022; Feng *et al*., 2022). The improved release of phenolic compounds contributed to the rise in antioxidant capabilities. The pre-treatments using a weak alkali solution could eliminate the condensed tannins up to 86.92% for 10 hours. Following a 60-hour fermentation process, the levels of condensed tannins were found to have decreased to 0.24%. In conclusion, the present study has demonstrated that fermentation can indeed reduce tannins, thus facilitating an increase in phenolic compounds and crude protein (Bahlawan *et al*., 2022; Chai *et al*., 2022; Feng *et al*., 2022).

Glutamic acid, arginine, and aspartic acids were the highest amino acids produced during the fermentation process of lotus tempeh. *Rhizopus oligosporus* produces protease which could degrade proteins into peptides and free amino acids. Furthermore, fungi are capable of facilitating the decomposition of primary substances, thereby generating derivatives or minor compounds, including volatile substances such as ammonia, aldehydes, and ketones. The volatile compounds are instrumental in determining the flavor of the resulting tempeh. The predominant substances in lotus seeds are carbohydrates, and it has been hypothesized that the degradation of these substances may result in the production of more acids. Profile's lotus tempeh, which is produced in optimal conditions, has been found to contain ethanol and naphthalene.

In the research by Kustyawati *et al.* (2017), pointed out that ordinary tempeh produced 23 volatile compounds consisting of alcohols (7 compounds), ketones (2 compounds), furans (2 compounds), fatty acids (4 compounds), esters (1 compound), hydrocarbons (1 compound), sesquiterpenes (2 compounds), benzenoids (3 compounds), and compounds containing sulfur (1 compound). As demonstrated in Table 5, lotus tempeh has 46 volatile compounds, with cyclobutanol and ethanol being the main compounds in profile's organic acids (Ridhowati *et al*., 2022). A tempeh produced from lotus and soybeans has been found to contain 19 identical compounds; cyclobutanol, isoamyl hydride, acetaldehyde, pentanal, ethyl acetate, ethanol, 3-pentanone, 2-butanol, 1-propanol, iso-butanol, 3-pentanol, iso-amyl acetate, limonene, iso-amyl alcohol, 2-pentyl-furan, iso-butenyl-carbinol, acetone, 1-hexanol, and phenylethyl alcohol. According to Kustyawati *et al.* (2017), acids easily evaporate because the long fermentation process produces a lot of acid. The distinctive aroma of tempeh is attributable to the growth of mold and the subsequent breakdown of components in the seeds into simple volatile compounds, including ammonia, aldehydes and ketones.

The presence of fungal mycelia has been observed on the surface of soybeans used in the production of tempeh. These mycelia have been shown to degrade components in the soybeans, resulting in the formation of a specific flavour profile during the fermentation process. Nainggolan *et al.* (2022) determined that the total BAL (*Bacteria Lactate Acid*) in lotus tempeh was higher than soybean tempeh; 6.58 log cfu/gr - 8.59 log cfu/gr, thus, the levels of acid and alcohol were found to exceed the established parameters. During fermentation, microorganisms produce enzymes to hydrolyze substrates into simple materials (sugar) and then convert them into ethanol. Several studies report that ethanol production is produced by microorganisms such as mold, yeast and bacteria. This yeast can grow in media containing simple sugars such as glucose, fructose and mannose. According to Hermanto *et al*. (2020), the presence of ethanol in food products during the fermentation process is attributable to the carbohydrate fermentation stage, which is catalyzed by enzymes to convert carbohydrates into glucose and subsequently into ethanol.

Lotus seeds are widely used in the production of fermented products and tempeh, which has a functional effect. The optimal condition for achieving the maximum protein content was determined using the RSM-Box Behnken method. This condition involved the use of 1 g of

starter, 15 hours of soaking time, and 36 hours of fermentation time, as well as 0.75 g of starter, 15 hours of soaking time, and 48 hours of fermentation time for the tannin content. Consequently, this research offers a promising basis for the efficient production of lotus tempeh, with the caveat that further development is required to explore the potential of alternative components as valuable protein and tannin sources. The research has concluded that the tempeh maker should choose the optimal condition with a high protein content due to tempeh being recognized as a protein source.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Sherly Ridhowati: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Herpandi Herpandi**: Definition of intellectual content, and manuscript review. **Indah Widiastuti**: Definition of intellectual content, and manuscript review.

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Research Article

The effect of inundation levels on secondary metabolites accumulation in *Avicennia marina* (Forsk.) roots under different salinity regimes

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Abstract: Salinity and inundation are factors that affect secondary metabolites. This research aims to study the range of typical secondary metabolite content in *Avicennia marina* growing at different salinity levels, analyze the level of inundation that causes peak stress, and examine the impact of inundation stress on *A. marina* under different salinity regimes. This study used a 2-factor factorial complete randomized design, namely salinity level (15, 20, 25, 30, and 35 ppt), and inundation level (10, 15, and 20 cm). The parameters measured were tannin content, total alkaloids, and total phenols in *A. marina* roots analyzed by spectrophotometry method. Data were analyzed by ANOVA and further tested with DMRT test. The concentration range of tannins, total alkaloids, and total phenols was 14.29–18.45%, 0.893–1.331 mgQE/g, and 62.7–8.75 mgGAE/g, respectively. Peak stress-induced by inundation in *A. marina* indicated by high secondary metabolite contents was differentiated based on the salinity regime. Peak secondary metabolite content was obtained from the combination of salinity and inundation of 25 ppt + 20 cm, 20 ppt + 15 cm, and 15 ppt + 10 cm for tannin, total alkaloid, and total phenol content with values of 18.26±0.17%; 1.301±0.021 mgQE/g; and 83.98±2.02 mgGAE/g. The research found that simultaneous effect of salinity and inundation impacted for all metabolites. Our result suggests that salinity has underlying effect on total alkaloid and total phenol concentration in *A. marina* roots, but not tannin. Inundation significantly affects tannin content, amplifying its effects on total alkaloid and total phenol content.

1. INTRODUCTION

Mangrove vegetation is an important component in the coastal area. One of the most important species among mangrove vegetation is *A. marina* acts as pioneer mangrove species (Naidoo & Naidoo, 2017) is frequently found in the newly developed mangrove ecosystem. It is also frequently used as preferred mangrove species for mangrove replanting in the degraded coastal area, such as in the flooded areas (van Bijsterveldt *et al*., 2022). As pioneer mangrove species, *A. marina* is able to sustain in the habitat with wide salinity variations and prolonged inundation (Etongo *et al*., 2022; Li, H., *et al*., 2020). Due to the dynamic environmental conditions, mangrove plants within mangrove ecosystem frequently undergo environmental stress (Limaye

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et al., 2014). The stress is typically induced by the hydrodynamic condition. This suggests that the development of mangrove ecosystem is different between geographic location.

Coastal area is the location where the ocean and land meets. Typically, it is characterized by saline environment. However, the salinity in the coastal area is different according to the geographic locations due to global sea water circulation (Liu & Wei, 2021). Additionally, local hydrological condition also have particular influence on the salinity of coastal waters, especially in the estuary areas (Pichler *et al*., 2017; Velmurugan *et al*., 2016). Local hydrology such as catchment area, land cover, as well as anthropogenic activities that lead to freshwater discharge is different among locations (Jiménez-Martínez *et al*., 2016; Li, B., *et al*., 2020). Therefore, due to the local hydrology alone, mangrove ecosystem in a location is considered as a specific unit.

Instead of the spatial variation, salinity in the marine areas also fluctuates temporally (Cloern *et al*., 2017; Shammi *et al*., 2017). Salinity would eventually go up and down gradually, forming a salinity fluctuation cycle (Liu & Wei, 2021). Temporal fluctuation of salinity is the result of seasonal cycle due to the change of freshwater supply and evaporation rate balance driven by the climate condition (Yu *et al*., 2020). It arises the question on how stress levels fluctuate in mangrove under different salinity regimes.

Instead of salinity, coastal areas are also characterized by the fluctuation of inundation level. The fluctuation of inundation is caused by tidal activities which cause the water surface to go high and low (Mawdsley *et al*., 2015). Due to tidal activities, the sea level continuously changes, while the changes can be seen hourly, daily and monthly (Anderson *et al*., 2021). The continuous fluctuation of sea level is the result of gravitational impact of the sun and the moon which changes due to the earth rotation (Haigh, 2017). Due to the everchanging environmental condition, mangrove plants in the coastal area are under continuous environmental pressures (Sofian *et al*., 2019). Due to salinity and inundation dynamics, mangrove habitat would never be in stable state, even for a day. However, the inundation fluctuation tends to have a greater role in inducing daily stress in mangrove since it fluctuates hourly, while daily salinity in the coastal area is typically uniform. Inundation is considered one of the most important aspects of mangrove survival, especially in the past decades. The vulnerability of mangrove toward inundation stress increases along with the occurring sea level rise (Di Nitto *et al*., 2014).

The everchanging environmental condition is suggested to have an impact on mangrove ecophysiological processes which is expressed in its secondary metabolites content (Yang *et al*., 2018). Mangrove plants typically produce various kind of secondary metabolites (Gajula *et al*., 2020). However, since the environmental condition of mangrove habitat significantly changes periodically, the secondary metabolites produced would change accordingly. High salt concentration causes various events that negatively impact. It has been reported that salt induced osmotic stress is responsible for the oxidative stress caused by reactive oxygen species (ROS) (Gengmao, *et al*., 2015). In response to such salinity stress, plant cells often produce a range of secondary metabolic like phenolic compounds. These phenolic compounds have been shown to be protecting biological systems against various oxidative stresses by playing a crucial role in the maintenance of redox homeostasis (Sadeghi, *et al*., 2024).

Understanding the impact of salinity and inundation on mangrove vegetation is important in order to properly manage mangrove ecosystems, especially for replantation. Under highly saline and frequent inundation, mangrove population is more vulnerable to possible collapse (Salmo & Juanico, 2015). Ambient salinity and inundation are important factors for individual mangrove development in the coastal area.

Referring to the previous explanation, the fluctuation of salinity level in the coastal area takes more time to show its significance. This research aimed to study the typical range of secondary metabolites content in *A. marina* growing in different salinity levels, to analyze the inundation level that causes *A. marina* to undergo peak stress, and to analyze stressability of inundation to *A. marina* growing in different salinity regimes.

2. MATERIAL and METHODS

2.1. Research Design

The research was conducted using an experimental approach. The experiment design was Completely Randomized Design with Factorial includes the application of different salinity and inundation levels. *A. marina* seedlings were planted in 80 L containers filled with mangrove soil and added with predefined saline water. The design for salinity variation was 15 ppt, 20 ppt, 25 ppt, 30 ppt and 35 ppt, while the design for inundation level was 10 cm, 15 cm, and 20 cm. The experiment was carried out in a greenhouse under ambient indirect lighting. The plant grew without direct sunlight. It was not exposed fully under the sunlight.

2.2. Sample Preparation

The plant used for the experiment was *A. marina* seedling. Planting media for *A. marina* seedling was a mix of mangrove mud and compost (2:1). Mangrove mud was retrieved from mangrove ecosystem located in Mangunharjo Village, Tugu District, Semarang City. Plastic barrel was used as planting container. The obtained media was placed in containers to the height of approximately 30 cm. *A. marina* seedling was then planted in the prepared media with density of 2 plants/barrel. Then, the barrel was filled with water with designed salinity to the designed inundation levels. The experiment was carried out for 60 days. Maintenance was carried out by adding saline water according to the treatment groups to maintain inundation level twice a week.

2.3. Determination of Total Tannin, Alkaloid, and Phenolic Components

Observation on secondary metabolites content was performed for mangrove roots. *Avicennia marina* roots were taken whole fully for laboratory analysis. Analysis of secondary metabolites content was performed quantitatively for tannin, total alkaloid and total phenol content using Spectrophotometry Method. UV/Vis double beam spectrophotometer and standard quarts cuvetts were used for all the absorbance measurement. Spectrofotometry methods are most commonly used for the quantification of tannin, alkaloid, and phenolic content (Tabasum, *et al*., 2016). The analysis procedure for each secondary metabolite according to Sarvade, *et al* (2020) and Tabasum, *et al* (2016) were:

a. Tannin

The sample was weighed as much as 50 mg, then 10 mL of diethyl ether was added to isolate the more polar tannin components, then the remaining diethyl ether was evaporated. 1 mL of sample solution was added 0.1 mL of Folin-Ciocalteu reagent. Added 2 mL of 20% sodium carbonate as a base buffer. The solution mixture was vortexed for 5 minutes so that all components react optimally. The color formed was then measured using a spectrophotometer with a wavelength of 760 nm. The next step is the preparation of a calibration curve with tannic acid as a standard. Tannic acid was weighed, then 10 mL of Folin-Ciocalteu reagent and 20% sodium carbonate was added. The color formed was then measured using a spectrophotometer with a wavelength of 760 nm. Tannin concentration can be determined by comparing the results with the standard calibration curve.

b. Alkaloid

Total alkaloid content was quantified by spectrophotometric method. This method is based on the reaction between alkaloid and bromocresol green (BCG). The plant extract (1 mg/ml) was dissolved in 2 N HCl and then filtered. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. 1 ml of this solution was transferred to a separating funnel, and then 5 ml of BCG solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extract was collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. The next step is the preparation of a calibration curve with quinine as a standard. Quinine was weighed as much as 10 mg, then the same steps starting from dissolving it in 2 N HCl, the pH of the phosphate

buffer solution was adjusted to neutral with 0.1 N NaOH, then 5 ml of BCG solution was added along with 5 ml of phosphate buffer. The mixture was shaken, and the complex formed was extracted with chloroform with vigorous shaking, then diluted to the same volume as the chloroform. Then the absorbance of each solution was taken at 470 nm using a spectrophotometer. The total alkaloid was determined with the help of a standard curve prepared from the quinine standard curve.

c. Phenol

The estimation of total phenol content in *A. marina* was measured spectrophotometrically with Folin-Ciocalteu reagent, using gallic acid as the standard and expresses the result as gallic acid equivalent (GAE) per gram of sample. The sample was weighed ± 50 mg, then 0.5 mL of Folin-Ciocalteu reagent was added. The reaction between phenol in the sample and the reagent produces a blue compound due to the oxidation process of phenol by the reagent. The blue color was measured spectrophotometrically at 765 nm. The next step is the preparation of a calibration curve with gallic acid as a standard. Gallic acid was weighed 10 mg, then added 0.5 mL of Folin-Ciocalteu reagent and 10 min later 1.5 mL of 20% sodium carbonate. Gallic acid standard solution was made with various concentrations (100; 75; 50; 25; 10; 5; 2.5; 1; 0.5; 0.2 ppm). After 10 minutes, the absorbance of each solution was taken at 765 nm using a spectrophotometer. The total phenol was determined with the help of a standard curve prepared from the pure phenolic standard curve (gallic acid).

2.4. Statistical Analysis

Data analysis was performed descriptively and statistically. Descriptive data analysis was performed through graphical illustration to represent laboratory analysis result of secondary metabolites content. Statistical analysis was performed through Analysis of Variance (ANOVA) with Factorial design to determine the effect of salinity and inundation on secondary metabolites content. Duncan's Multiple Range Test (DMRT) to further test.

3. RESULTS

The content of secondary metabolites varied throughout the research. Referring to the obtained data, average tannin content was highest (16.87%) under salinity of 30 ppt, but the variation was greatest in 25 ppt (14.29% – 18.45%). The average total alkaloid content on the other hand, was found highest in 20 ppt (1.224 mgQe/g), but the variation was greatest in 35 ppt (0.893 - 1.327 mgQE/g). The average total phenol content was highest of 15 ppt (79.35 mgQE/g), while the variation was greatest at 35 ppt (65.83-84.57 mgQe/g). Detailed laboratory analysis result of secondary metabolites content studied in this research is presented in Table 1. The highest content of tannin, alkaloid, and phenol was found in different salinity level.

Table 1. Secondary metabolites content in *A. marina* roots grown under different salinity regimes.

Salinity (ppt)	Secondary Metabolite Content		
	Tannin (%)	Total Alkaloid (mgQE/g)	Total Phenol (mgGAE/g)
15	14.70-17.44	0.899-1.323	72.55-85.65
	16.28±0.89[a]	1.086±0.122[a]	79.35±4.36[c]
20	14.97-17.47	1.126-1.331	70.19-76.70
	16.09±0.79[a]	1.224±0.071[a]	73.25±2.00[a]
25	14.29-18.45	1.023-1.282	62.77-74.36
	16.67±1.53[a]	1.157±0.092[a]	68.67±3.68[b]
30	15.60-18.33	1.006-1.301	68.30-80.42
	16.87±0.81[a]	1.177±0.123[a]	75.67±3.66[b]
35	14.90-18.27	0.893-1.327	65.83-84.57
	16.76±0.99[a]	1.132±0.166[a]	76.11±5.95[bc]

Note: different letter within the same column indicates significant difference

The research found that *A. marina* response to inundation stress changes according to the salinity setting. Detailed result on the response of *A. marina* toward inundation stress under different salinity regimes are showed in Figure 1 to Figure 3.

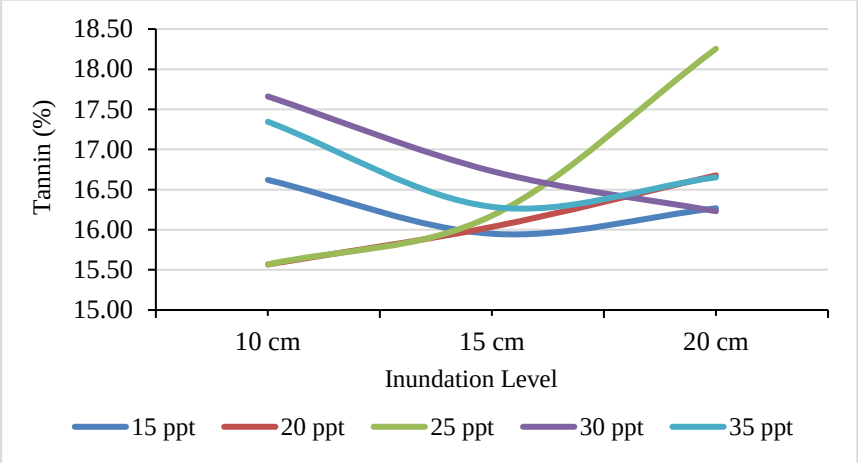

Figure 1. Tannin content of *A. marina* roots in response to the variation of inundation levels under different salinity levels.

Figure 1 shows the experiment result on tannin concentration in *A. marina* roots. Figure 1 shows, tannin concentration responded differently toward inundation stress under different salinity regime. Tannin concentrations were highest in the inundation level of 10 cm for *A. marina* grown in 15 ppt, 30 ppt and 35 ppt salinity levels. While 20 cm of inundation for those grown in the salinity of 20 ppt and 25 ppt. Refer to Figure 1, under salinity of 20 ppt and 25 ppt, there was the description of trends along with the increase of inundation levels to tannin content and a decreased effect trend of inundation level under salinity of 30 ppt. While under salinity of 15 ppt and 35 ppt, the trends of inundation effect have parabolic pattern. Refer to the finding of the result, peak tannin content in *A. marina* roots were 16.62 ± 0.46 %, 16.68 ± 0.73 %, 18.26 ± 0.17 %, 17.66 ± 0.52 %, and 17.35 ± 0.95% respectively for the salinity of 15 ppt, 20 ppt, 25 ppt, 30 ppt and 35 ppt. This suggests that under the salinity of 25 ppt, tannin content in *A. marina* roots was highest compared to other salinity levels.

Statistical analysis with Factorial ANOVA showed F value of 2.981 with probability of 0.003 which indicates that there was a simultaneous effect between salinity and inundation levels to tannin content in *A. marina* roots. However, there was no partial effect of salinity and inundation, showed by the F value of 1.783 and 2.274 with the probability of 0.149 and 0.115 respectively.

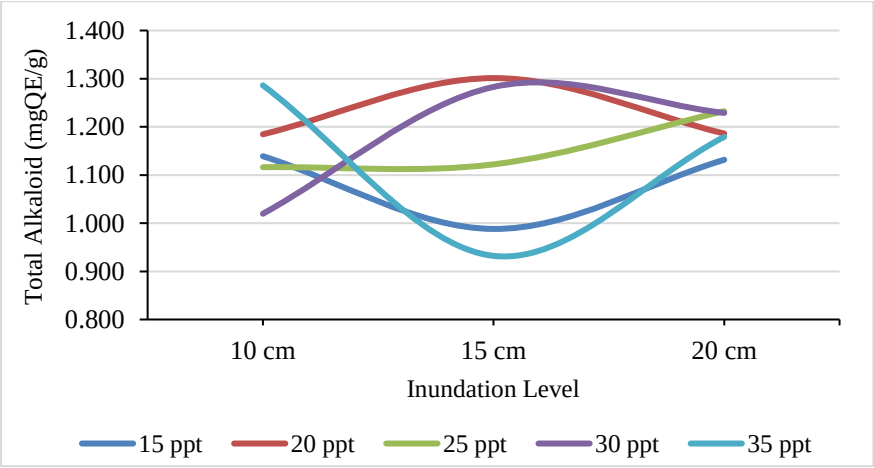

Figure 2. Total alkaloid content of *A. marina* roots in response of the variation of inundation levels under different salinity levels.

Different reaction of *A. marina* toward inundation stress under different salinity regimes was also shown by its total alkaloid content. The analysis result as presented in Figure 2 shows that the fluctuation of total alkaloid content toward inundation stress typically have parabolic pattern. Refer to the result, total alkaloid content was highest in 10 cm inundation for *A. marina* grown under 15 ppt and 35 ppt, 15 cm for those grown under 20 and 30 ppt, and 20 cm for those grown under 25 ppt. Refer to the analysis result, peak total alkaloid content in respective salinity levels is 1.139 ± 0.129 mgQE/g, 1.301 ± 0.021 mgQE/g, 1.233 ± 0.071 mgQE/g, 1.283 ± 0.016 mgQE/g and 1.286 ± 0.040 mgQE/g.

Statistical analysis with Factorial ANOVA showed that there was a significant simultaneous effect of salinity and inundation to total alkaloid concentration. The significance of simultaneous effect was showed by its F value and probability which were 9.915 and 0.000 respectively. The effect of salinity and inundation on total alkaloid was also observed in partial model. The effect of salinity was showed by its F value of 6.021 and probability of 0.001, while the effect on inundation was showed by its F value of 4.330 and probability of 0.019.

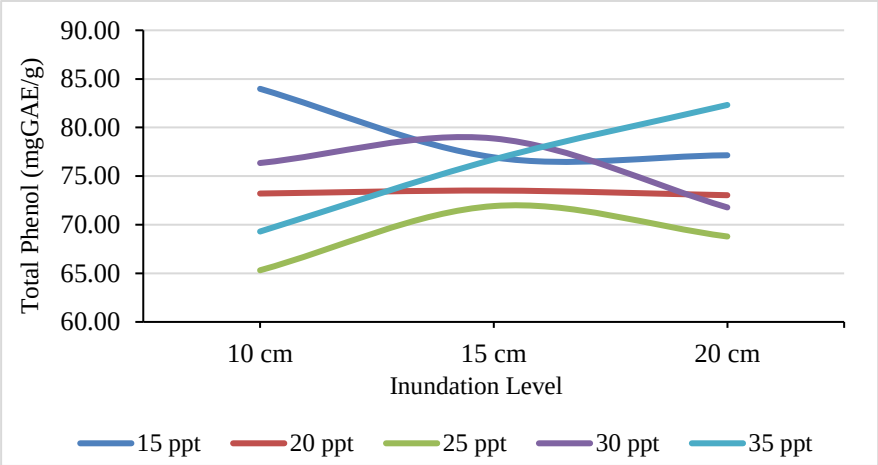

Figure 3. Total phenol content of *A. marina* roots in response of the variation of inundation levels under different salinity levels.

Based on statistical test with ANOVA was performed to emphasize the difference of peak secondary metabolites content induced by inundation stress at each salinity level. Figure 3 shows the response of *A. marina* toward inundation stress under different salinity regimes expressed by total phenol content. Various effect pattern between inundation level and total phenol content was obtained from the observation, including parabolic for 15 ppt, 25 ppt and 30 ppt and linear for 20 ppt and 35 ppt. Refer to the result, total phenol content in *A. marina* roots was highest in inundation level of 10 cm under 15 ppt salinity. While in 15 cm of inundation level, total phenol content was highest under salinity of 20 ppt, 25 ppt and 30 ppt. Lastly, under 35 ppt of salinity, total phenol content was highest in 20 cm of inundation. Refer to the analysis result, peak total phenol content in *A. marina* roots grown under salinity of 15 ppt, 20 ppt, 25 ppt, 30 ppt and 35 ppt are 83.98 ± 2.02 mgGAE/g, 73.52 ± 2.42 mgGAE/g, 71.92 ± 1.84 mgGAE/g, 78.87 ± 1.35 mgGAE/g and 82.32 ± 2.00 mgGAE/g.

Statistical analysis for total phenol concentration also indicated the significance of salinity and inundation effects, both partially and simultaneously. The significance of simultaneous effect was showed by its F value and probability of 16.395 and 0.000. Partially, statistical analysis for the salinity effect showed the value of 30.601 and 0.000 respectively, while the effect of inundation was showed by the value of 3.118 and 0.054 respectively for F and probability.

In order to further understand the distribution of peak secondary metabolites concentration in regards of the inundation levels, an extraction of values from each salinity group. Additionally, a statistical test with ANOVA was performed to emphasize the difference of peak secondary metabolites content induced by inundation stress at each salinity level. The comparison between inundation level and secondary metabolites content is presented in Figure 4 to Figure 6.

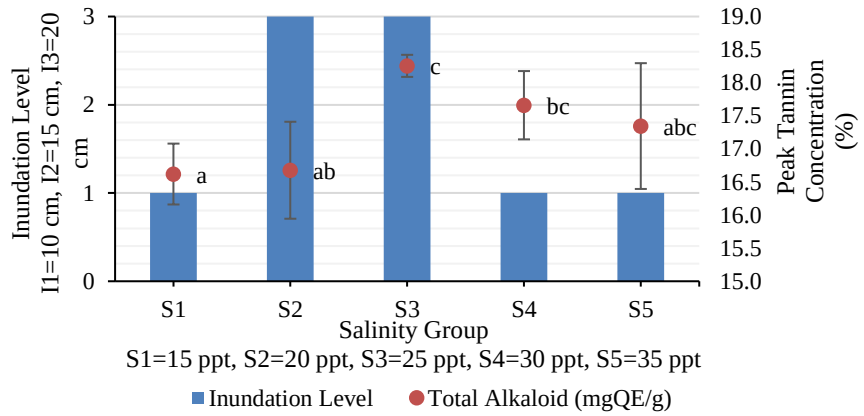

Figure 4. Peak tannin concentration in *A. marina* roots in accordance with inundation level at each salinity regime; S is salinity level.

Figure 4 shows the comparison between inundation levels and tannin content in *A. marina*. The result shows that tannin concentration was highest under salinity of 25 ppt and inundation level of 20 cm. Statistical analysis showed that peak tannin concentration in *A. marina* roots under salinity of 25 ppt was significantly higher than those below 25 ppt. Statistical analysis with ANOVA showed the F value of 4.864 and probability of 0.010.

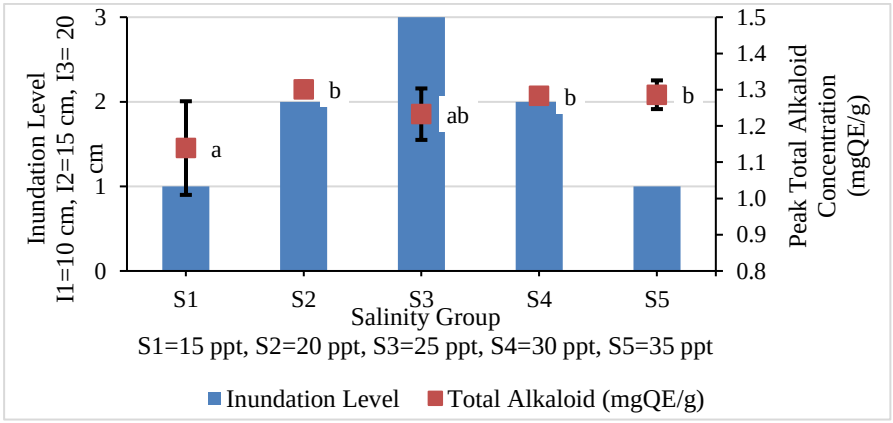

Figure 5. Peak total alkaloid concentration in *A. marina* roots in accordance with inundation level at each salinity regime.

Figure 5 shows the comparison between inundation levels and total alkaloid content in *A. marina* root. The result shows that total alkaloid content was highest under salinity of 20 ppt and inundation level of 15 cm which difference is insignificant to other treatments with higher salinity levels, but significant to lower salinity level. Statistical analysis with ANOVA showed the F value of 3.671 and probability of 0.028.

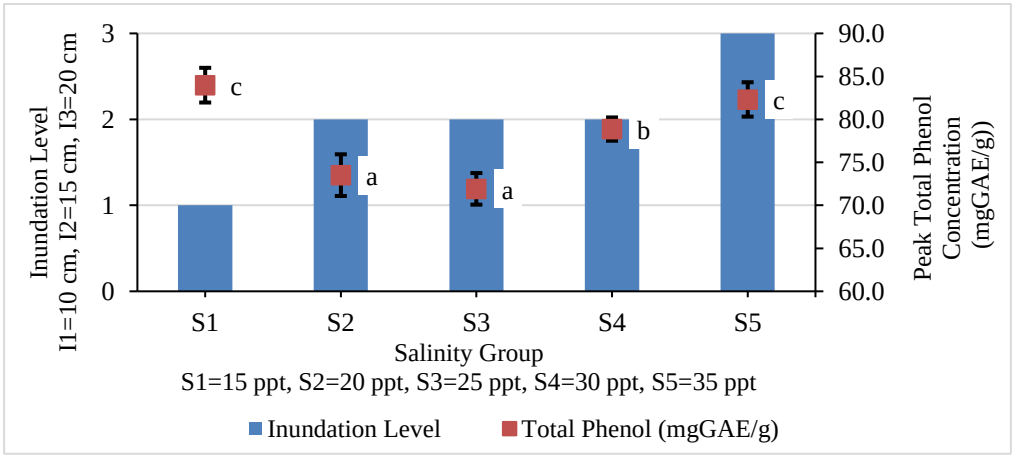

Figure 6. Peak total phenol concentration in *A. marina* roots in accordance with inundation level at each salinity regime.

Figure 6 shows the comparison between inundation levels and total phenol content in *A. marina* root. The result shows that total phenol content was highest under the salinity of 15 ppt and inundation level of 10 cm which content is indifferent from the treatment of 35 ppt salinity and 20 cm inundation, but different other salinity levels in between. Statistical analysis with ANOVA showed an F value of 29.288 and a probability of 0.000.

Further analysis was performed through ANOVA to differentiate the secondary metabolites concentration stored in *A. marina* roots under different salinity regimes and inundation levels. Univariate statistic test showed that there was significant combined effect of inundation and salinity to tannin, total alkaloid, and total phenol content in *A. marina* roots.

Univariate statistic test on tannin content showed F value of 2.981 and probability of 0.003, which indicates that the combination effect between inundation and salinity was significant. However, salinity that is considered as the predetermined condition, did not have an effect on the variation of tannin content. Therefore, inundation levels are considered as the factor that signifies the variation of tannin content. Referring to the analysis result, the highest tannin concentration was observed in the treatment of 25 ppt salinity and 20 cm inundation, while the lowest was obtained from 25 ppt salinity and 10 cm inundation. Detailed analysis result on tannin content is presented in Table 2.

Table 2. Homogenous subsets of tannin content in *A. marina* roots under different salinity and inundation levels.

Salinity	Inundation Levels		
	10 cm	15 cm	20 cm
15 ppt	16.62 ± 0.46^{abc}	15.95 ± 1.11^{ab}	16.27 ± 1.08^{abc}
20 ppt	15.57 ± 0.53^{a}	16.04 ± 0.79^{ab}	16.68 ± 0.73^{abc}
25 ppt	15.57 ± 1.16^{a}	16.18 ± 1.38^{ab}	18.26 ± 0.17^{d}
30 ppt	17.66 ± 0.52^{cd}	16.73 ± 0.38^{abc}	16.23 ± 0.76^{abc}
35 ppt	17.35 ± 0.95^{bcd}	16.29 ± 0.74^{abc}	16.65 ± 1.18^{abc}

Univariate statistic test on total alkaloid content showed F value of 9.195 and probability of 0.000, which indicates that the combination effect between inundation and salinity was significant. According to the analysis result, salinity has particular effect on the variation of total alkaloid content. Therefore, inundation levels is considered as the amplifying factors of the variation of total alkaloid content in *A. marina* roots. Referring to the analysis result, the highest total alkaloid content was obtained from the treatment of 20 ppt salinity and 15 cm inundation, while the lowest was obtained from 35 ppt salinity and 15 cm inundation. Detailed analysis result on total alkaloid content is presented in Table 3.

Table 3. Homogenous subsets of total alkaloid content in *A. marina* roots under different salinity and inundation levels.

Salinity	Inundation Levels		
	10 cm	15 cm	20 cm
15 ppt	1.139 ± 0.129^{c}	0.988 ± 0.061^{a}	1.132 ± 0.122^{c}
20 ppt	1.185 ± 0.038^{cde}	1.301 ± 0.021^{e}	1.186 ± 0.068^{cde}
25 ppt	1.116 ± 0.049^{bc}	1.122 ± 0.110^{bc}	1.233 ± 0.071^{cde}
30 ppt	1.020 ± 0.012^{ab}	1.283 ± 0.016^{de}	1.229 ± 0.060^{cde}
35 ppt	1.286 ± 0.040^{de}	0.932 ± 0.049^{a}	1.179 ± 0.095^{cd}

Univariate statistic test on total phenol content showed F value of 16.395 and probability of 0.000, which indicates that the combination effect between inundation and salinity was significant. According to the analysis result, salinity particularly has a significant effect on total

phenol content in *A. marina* roots. This suggests that the inundation levels act as the amplifier of total phenol content differentiation. Referring to the analysis result, the highest total phenol content was obtained from the treatment of 15 ppt salinity and 10 cm inundation, while the lowest was obtained from 25 ppt salinity and 10 cm inundation. Detailed analysis result on total phenol content is presented in Table 4.

Table 4. Homogenous subsets of total phenol content in *A. marina* roots under different salinity and inundation levels.

Salinity	Inundation Levels		
	10 cm	15 cm	20 cm
15 ppt	83.98 ± 2.02^{i}	76.93 ± 1.87^{efg}	77.15 ± 4.39^{fg}
20 ppt	73.21 ± 2.05^{cdef}	73.52 ± 2.42^{def}	73.04 ± 2.09^{cde}
25 ppt	65.32 ± 2.31^{a}	71.92 ± 1.84^{bcd}	68.79 ± 3.46^{ab}
30 ppt	76.35 ± 2.35^{efg}	78.87 ± 1.35^{gh}	71.78 ± 2.71^{bcd}
35 ppt	69.3 ± 2.89^{bc}	76.72 ± 1.89^{efg}	82.32 ± 2.00^{hi}

The result suggests that tannin content, total alkaloid content and total phenol content react differently toward salinity and inundation levels. Inundation levels could act as the signifying or amplifying factors of environmental stressors to *A. marina*.

4. DISCUSSION and CONCLUSION

Salinity and inundation are two major factors corresponding to stress in mangrove vegetation (Salmo & Juanico, 2015). In the coastal area, both variables are known to fluctuate in different cycles and the intersection creates unique ecological condition of mangrove ecosystem. According to Perri *et al*. (2017) the combination of salinity and inundation in mangrove ecosystem plays important role in determining mangrove's ability regarding water uptake, photosynthesis, stomatal conductance, gas exchange and nutrient availability. Referring to the finding of this research, salinity variation has partial impact on total alkaloid and total phenol content, but not on tannin content. Salinity is the main stress drivers to mangrove plants in the coastal area (Alhassan & Aljahdali, 2021). The salinity issue exists not only during the flooding period, but also prolonged to non-flooding period. Less-flooded mangrove ecosystem tend to have a saline sediment (Wunderlich & Pinheiro, 2013). Thus, even after land establishment, mangrove ecosystem will remain saline.

The research suggests that tannin content in *A. marina* root is not significantly affected by environment's salinity variation. Typically, tannin is an important metabolites that helps mangrove plants to cope with salinity stress (Zhu *et al*., 2023). Therefore, this finding suggests that the designed salinity variation (15 – 35 ppt) is still within the tolerance range of *A. marina*. However, tannin content in *A. marina* seedling during the experiment was signified by the inundation. Signified tannin content difference by inundation variation suggests that inundation amplifies the magnitude of stress factors related to tannin synthesis. Refer to Cui *et al*. (2022), tannin concentration in mangrove plants is related to biological stress such as bacteria. In addition, the microbial community in the mangrove ecosystem is more influenced by inundation than salinity (Chambers *et al*., 2016).

Total alkaloid content and total phenol content on the other side was also significantly partially affected by salinity variation. There was no particular trend of salinity's effect on total alkaloid content. However, a parabolic trend resulted in total phenol content. High total phenol content was obtained from the treatment with 15 ppt and 35 ppt of salinity, and parabolically decreased in between. According to Yang *et al*. (2018), alkaloid and phenol content in mangrove is related to temperature stress. However, Pant *et al*. (2021) found that the fluctuation of total alkaloid and total phenol contents are also related to other factors, such as light intensity, drought, salinity and soil fertility. Other than physical and chemical factors, total phenol content could

also be related to biological factors such as fungi abundance. According to Luo *et al*. (2018), under fungal disturbance, mangrove plants may excrete more phenol, causing higher availability of phenol in the sediment. In addition to the salinity factor, inundation also contributes to the amplification of total alkaloid and total phenol content in *A. marina* roots. Inundation may add other stressing factors to salinity variation. According to Li *et al*. (2020), inundation is important factor that lead to metabolic modification. Therefore, the ability of *A. marina* to sustain inundation comes with the cost of decreased photosynthesis rate.

Variation of tannin, total alkaloid and total phenol content in *A. marina* roots showed that mangroves undergo different pressures of combined salinity and inundation level. Accumulation of secondary metabolites is the mechanism of mangrove to improve its tolerance toward environmental pressures (Ravi *et al*., 2020). According to a research by Barnuevo and Asaeda (2018), the combination of salinity and inundation levels affect the development of mangrove plants both in short and long periods such as first leaf development and total height gain.

The finding of this research suggests that mangroves, especially *A. marina*, reacts differently toward the variation of salinity and inundation combination. This finding is important to determine which environmental condition is less stressful and which one is more stressful to *A. marina*. Therefore, a proper management strategy in nursery of mangrove breed could be applied. The finding is also important in case of the utilization plan of mangrove as source of secondary metabolites extract. According to Hurmat *et al*. (2020), environmental modification is frequently applied in the cultivation of medicinal plants. It is expected to provide metabolites products in a greater sum and/or better quality.

Secondary metabolites content in *A. marina* roots under varied environmental settings were between 14.29 to 18.45 % for tannin, 0.893 to 1.331 mgQE/g for total alkaloid and 62.77 to 85.65 mgGAE/g for total phenol. Peak stress induced by inundation in *A. marina* indicated by high secondary metabolites content was differentiated by salinity regimes, including 10 cm of inundation under 15 ppt, 30 ppt and 35 ppt salinity and 20 cm of inundation under 20 ppt and 25 ppt salinity for tannin content, 10 cm of inundation under 15 ppt and 35 ppt salinity, 15 cm under 20 ppt and 30 ppt salinity and 20 cm under 25 ppt salinity for total alkaloid content, as well as 10 cm under 15 ppt salinity, 15 cm under 20 ppt, 25 ppt and 30 ppt salinity, and 20 cm under 35 ppt salinity for total phenol content. Inundation plays an important role in inducing stress to *A. marina*, proved by the ability of inundation to signify and amplify the effect of salinity on the variation of secondary metabolites content.

In response in salinity and inundation stress, *Avicennia* increases the production of secondary metabolites like tannins, alkaloids, and phenolics, which act as antioxidants to detoxify ROS and protect cellular structures from oxidative damage. Secondary metabolites also helping to maintain cellular turgor and prevent dehydration. Stress hormones like abscisic acid (ABA) and ethylene are key regulators of secondary metabolite pathways. According to Reshi, *et al* (2023), salinity and inundation both trigger ABA accumulation, which stimulates the synthesis of secondary metabolites. Inundation increases ethylene production which can further activate stress response pathways, amplifying the effects of salinity on secondary metabolite production.

The conclusion of this research is the range of secondary metabolite content in *A. marina* has a different range for each metabolite. Tannin has the greatest variation in 25 ppt (14.29% – 18.45%), alkaloid in 35 ppt (0.893 - 1.327 mgQE/g), while phenol in 35 ppt (65.83-84.57 mgQe/g). The inundation level that causes *A. marina* to undergo peak stress was found based on peak total metabolite concentration. The highest tannin concentration was observed in the treatment of 25 ppt salinity and 20 cm inundation, alkaloid highest concentration was obtained from 35 ppt salinity and 15 cm inundation, while phenol highest concentration was found in 25 ppt salinity and 10 cm inundation. These findings highlight the amplifying role of inundation in the salinity-induced stress response of *A. marina*. The results have important implications for mangrove management strategies and the utilization of *A. marina* as a valuable source of

secondary metabolites. Further studies are needed to elucidate the biochemical mechanisms underlying these responses and to explore the performance of *A. marina* under more complex natural environmental conditions.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Endah Dwi Hastuti: Organized research, Conception, Data collection and processing, Writing publication manuscript and report. **Erma Prihastanti**: Data collection, Laboratory sample testing, Analysis and interpretation.

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Research Article

Exploring *Phlomis crinita* extracts: HPLC analysis, phenolic content, antioxidant and antimicrobial potentials

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Abstract: *Phlomis crinita* Cav. (*P. crinita*), known as " Khayat el-djerah " in Algerian folk medicine, is used for wound healing and abdominal pain relief. This study assessed the phytochemical profile, phenolic content, antimicrobial activity against five Gram-negative and three Gram-positive clinical bacterial strains, as well as *in vitro* antioxidant activity of hydroethanolic extracts from leaves (HLE), flowers (HFE), and rhizomes (HRE) of *P. crinita*. Fifteen phenolic compounds such as four flavonoids, trans-cinnamic acid, six cinnamic acid derivatives, and four benzoic acid derivatives were identified for the first time in *P. crinita* by HPLC-DAD, with quantitative differences among the analyzed parts. HRE exhibited high levels of total phenolics (262.97 ± 16.2 µg GAE/mg DW) and flavonoids (71.87 ± 3.25 µg QE/mg DW), while HLE had the highest flavonols content (18.89 ± 5.12 µg QE/mg DW). All extracts demonstrated strong antioxidant properties. HLE exhibited the highest potency, with IC_{50} values of 15.46 ± 0.45 µg/mL (DPPH) and 11.71 ± 0.50 µg/mL (ABTS). HLE exhibited good reducing power (FRAP $A_{0.5}$ = 40.07 ± 2.82 µg/mL), while HRE showed the best reducing power (Phenanthroline $A_{0.5}$ = 7.88 ± 1.63 µg/mL). All extracts revealed broad-spectrum antibacterial effects, and HRE exhibited the most potent activity against *Enterococcus faecalis*, with a minimum inhibitory concentration (MIC) value of 1.25 mg/mL. These results showed that *P. crinita* could be useful as source of bioactive compounds for pharmaceutical and food industry.

1. INTRODUCTION

Medicinal plants play a crucial role in traditional and modern healthcare, providing a wealth of bioactive compounds with therapeutic potential. In fact, natural products play a pivotal role as a source of drug compounds, and currently, a number of modern drugs derived from traditional herbal medicine are used in modern pharmacotherapy (Marrelli, 2021). Recently, there has been

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a growing interest in conducting phytochemical and pharmacological studies on traditional medicinal plants. The Lamiaceae family, a well-known source of bioactive molecules, has attracted significant attention. Among this family, the genus *Phlomis*, encompassing over 100 species of perennial herbs, is primarily found in regions around 40° north latitude including Algeria, Morocco, Spain, Greece, Turkey, and China (Li *et al*., 2010). Previous research has demonstrated that the genus *Phlomis* is rich in secondary metabolites, including flavonoids, phenolic acids, iridoids, phenylethylalcohol glycosides, phenylethanoid and benzyl glycosides, lignans, and terpenoids, and most of these classes belong to the polyphenol category and serve as powerful antioxidants due to their multiple hydroxyl groups (Amor *et al*., 2009a; Li *et al.*, 2010; Çalış & Başer, 2021). This has led to the exploration of the phytochemical and pharmacological properties of various *Phlomis* species, which have shown promise in the treatment of many illnesses. In fact, biological investigations of extracts of the genus *Phlomis* have demonstrated their diverse range of activities, including anti-inflammatory, immunomodulatory, free-radical scavenging, antimicrobial, anti-mutagenic, and anti-ulcerogenic effects (Limem *et al*., 2011).

Phlomis crinita Cav. (synonym: *P. biloba*), one of *Phlomis* species found in Algeria, is locally known as "Khayat el djerah" (wound healer) due to its traditionally used leaves for treating lesions and burns (Amor *et al*., 2009a; Boutennoun *et al*., 2023). Despite its ethnomedicinal significance, the phytochemical profile of *P. crinita* remains largely unexplored, with only a few studies examining its chemical composition. Given its traditional medicinal applications, there is a pressing need to investigate its phenolic profile and associated biological activities.

This study aimed to evaluate the phenolic composition, total phenolic content, flavonoid content, flavonol content, antioxidant capacity, and antibacterial efficacy of hydroethanolic extracts from the leaves (HLE), flowers (HFE), and rhizomes (HRE) of *P. crinita*. Specifically, the objectives were to: (1) identify and quantify phenolic compounds using high-performance liquid chromatography with diode array detection (HPLC-DAD); (2) determine total phenolic, flavonoid, and flavonol contents via spectrophotometric assays; (3) assess antioxidant activity using four methods: DPPH• free radical scavenging, ABTS•+ cationic radical scavenging, Ferric Reducing Antioxidant Power (FRAP), and the Phenanthroline reduction method; and (4) evaluate antibacterial activity against clinical bacterial strains using the broth microdilution method.

2. MATERIAL and METHODS

2.1. Chemicals

High-quality, analytical-grade chemicals and solvents were used in this study, including: aluminum chloride, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ascorbic acid, α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), chloroform, 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide, ethanol, ferric chloride, Folin-Ciocalteu reagent, gallic acid, hydrochloric acid (HCL), iodine, magnesium turnings, mercuric chloride, potassium bismuth iodide (Dragendorff's reagent), potassium acetate, potassium ferricyanide, potassium iodide, potassium persulfate, quercetin, sodium acetate, and trichloroacetic acid (Sigma-Aldrich, St. Louis, MO, USA). Sodium carbonate, ferric chloride, and phenanthroline were obtained from Biochem Chemopharma. Sodium hydroxide was obtained from PanReac AppliChem. Sulfuric acid was purchased from Honeywell International Inc. All chemicals and reagents were used without further purification.

2.2. Plant Material

After botanical identification of the species *P. crinita* by Professor Abdelkader Saadi (Faculty of Natural and Life Sciences, University of Chlef), fresh leaves, flowers, and rhizomes were collected during the flowering phase in June 2022 from a locality called Sidi Amer, located within Chlef province in the north-west of Algeria (latitude: 36°11'10.3"N, longitude:

1°18'18.2"E). The plant parts were removed from the stems, air-dried at room temperature for 15 days while avoiding exposure to direct sunlight, and then ground into a fine powder using a blender. The rhizomes were ground separately using a mortar and pestle. The resulting powders were kept in opaque containers at ambient temperature (25°C) until subsequent use.

2.3. Preparation of Extracts

The extraction was performed according to the method described by Merouane *et al*. (2020) with minor modifications. 10 g of fine powder from each sample (leaves, flowers, and rhizome) were macerated in 200 mL of ethanol (80%) at ambient temperature (25°C), while being shaken continuously for 24 hours using a WIS-10 shaking incubator (Daihan Scientific Co. Ltd., Korea). Subsequently, the mixtures were filtered through Whatman No. 1 filter paper, and the residues were rinsed twice with 10 mL aliquots of 80% ethanol. The resulting filtrates were then concentrated using a rotary evaporator (Büchi, Flawil, Switzerland) at temperatures below 48 °C under reduced pressure to remove ethanol. and then dried in an oven at 35 °C for 48 h to remove any remaining water. Each crude extract was then weighed, and the yield was calculated. Finally, the extracts were stored in a refrigerator at 4 °C until use for investigation.

The yield (%) of soluble constituents obtained through the hydroethanolic extraction was quantified using the equation below to assess extraction efficiency:

$$\% \text{ Yield} = (\text{Mass of the dried extract in mg} / \text{Initial mass of the sample in mg}) \times 100 \quad \text{(Eqn. 1)}$$

2.4. Qualitative Phytochemical Screening

Several standard tests were employed to highlight major phytoconstituents in the extracts, using methods described by Iqbal *et al*. (2015) and Saptarini *et al*. (2016).

2.4.1. *Tests for flavonoids*

A 1 g sample was extracted with 10 mL of 70% ethanol and filtered.

a. Shinoda test: a few turnings of magnesium and concentrated HCL (5 drops) were added to the ethanolic extract. The appearance of red to pink color after few minutes indicates the presence of flavonoids (Saptarini *et al*., 2016).

b. NaOH test: A few drops of 1N sodium hydroxide solution were added to the ethanolic extract. An intense yellow color which disappeared after adding dilute HCL indicates the presence of flavonoids (Saptarini *et al*., 2016).

2.4.2. *Test for terpenoids*

a. Salkowski test: approximately 100 mg of the crude extract was shaken with 2 mL of chloroform. Subsequently, 2 mL of concentrated sulfuric acid was added along the side of the test tube. The formation of a reddish-brown coloration at the interface indicates the presence of terpenoids (Iqbal *et al*., 2015).

2.4.3. *Test for tannins*

a. Ferric chloride test: A separate extraction was conducted for leaf, flower and rhizome samples, each weighing 0.5 g. These samples were individually mixed with 10 mL of distilled water and subjected to stirring, followed by filtration to obtain the aqueous extracts. Subsequently, a few drops of 5% ferric chloride solution were introduced to each filtrate. A black or blue-green coloration or precipitate was taken as a positive result for the presence of tannins (Iqbal *et al.*, 2015).

2.4.4. *Tests for alkaloids*

A few milligrams (about 15 mg) of each extract were separately stirred with 1% HCL (6 mL) in a water bath for 5 minutes and filtered. These filtrates were divided into three equal parts.

a. Dragendorff's test: A 1 mL aliquot of Dragendorff's reagent, a solution of potassium bismuth iodide, was introduced to a portion of the filtrate. The formation of an orange-red precipitate indicates the presence of alkaloids (Iqbal *et al*., 2015).

b. Mayer's test: To another aliquot of the filtrate, 1 mL of Mayer's reagent, composed of potassium mercuric iodide, was added. The subsequent formation of a cream-colored precipitate serves as an indicator for the presence of alkaloids (Iqbal *et al*., 2015).

c. Wagner's test: A solution was prepared by dissolving 2 g of potassium iodide and 1.27 g of iodine in 5 mL of distilled water and subsequently diluting this mixture to 100 mL with additional distilled water. Upon adding several drops of this reagent to the filtrate, the appearance of a brown precipitate confirms the presence of alkaloids (Iqbal *et al*., 2015).

2.5. Analyses of Phenolic Compounds (HPLC)

The method described by Fedoul *et al.* (2022) was used for HPLC analysis of phenolic compounds in *P. crinita* hydroethanolic extracts. An HP-Agilent 1290 Infinity HPLC system, equipped with a 250×4.6 mm C18 ultrasphere-ODS column and a diode array detector (DAD), was used with a mobile phase consisting of 3% acetic acid in water (A) and methanol (B). Samples (20 mg/mL) prepared in methanol were injected in 10 µL and detected at 278 nm. The elution gradient applied at a flow rate of 0.8 ml/min was: 93% A-7% B (0.1 min), 72% A-28% B (20 min), 75% A-25% B (8 min), 70% A-30% B (7 min) and the same gradient for 15 min was 67% A-33% B (10 min), 58% A-42% B (2 min), 50% A-50% B (8 min), 30% A-70% B (3 min), 20% A-80% B (2 min) and 100% B in 5 min until the end of the experimental cycle. Identification and quantification of phenolic compounds were achieved by comparison with standard phenolic compounds (Table 2). External calibration curves prepared for each standard were used to express individual phenolic component amounts in ppm (mg/kg extract).

2.6. Quantifying Phenolics, Flavonoids, and Flavonols

2.6.1. *Total phenolic content (TPC)*

TPCs of the extracts (HLE, HFE and HRE) were estimated with the method of Müller *et al*. (2010). Briefly, 20 µL of each extract (1 mg/mL) or gallic acid standard solution was mixed with 100 µL of Folin–Ciocalteu reagent. Then, 75 µL of sodium carbonate solution (7.5%) was added to the mixture. Following a 2-hour incubation in darkness, the reaction mixtures were assessed for absorbance at 765 nm with a microplate reader (Perkin Elmer, EnSpire, Singapore). TPC was then calculated against a gallic acid calibration curve (r^2 = 0.997) and expressed as µg gallic acid equivalents (GAE)/mg dry weight.

$$\text{TPC (µg GAE/mg dry weight)} = C_{GA} \times \text{Vol} / \text{Mass} \quad \text{(Eqn. 2)}$$

Where C_{GA}, Vol, and Mass are the concentration of gallic acid established from the calibration curve (µg/mL), the volume of the extract (mL) used in the assay, and the weight of the extract (mg) used in the assay, respectively.

2.6.2. *Total flavonoid content (TFC)*

TFCs of extracts (HLE, HFE and HRE) were quantified using a microplate assay adapted from the method described by Benouchenne *et al*. (2020). Briefly, 50 µL of each extract (1 mg/mL) or quercetin standard solution was combined with 130 µL of methanol, 10 µL potassium acetate solution (9.8%) and 10 µL of aluminium nitrate solution (10%), and mixed. After incubating for 40 minutes, the absorbance of the reaction mixtures was measured at 430 nm using a microplate reader (Perkin Elmer, EnSpire, Singapore). TFC was then calculated relative to a calibration curve constructed with quercetin as the standard (r^2 = 0.997) and expressed in µg quercetin equivalents (QE)/mg dry weight.

$$\text{TFC (µg QE/mg dry weight)} = Cq \times \text{Vol} / \text{Mass} \quad \text{(Eqn. 3)}$$

Where Cq, Vol, and Mass are the concentration of quercetin obtained from the calibration curve (µg/mL), the volume of the extract used in the assay (mL), and the weight of the extract (mg) used in the assay, respectively.

2.6.3. *Total flavonol content (TFoC)*

The aluminium trichloride ($AlCl_3$) colorimetric approach was slightly modified for microplate determination in order to assess the TFoCs (Bendjedid, *et al*., 2020). A mixture of 50 µL of each extract (HLE, HFE and HRE) (1 mg/mL), 50 µL of aluminium trichloride solution (2%), 150 µL of sodium acetate solution (5%) was added. After the mixture was left in the dark for 150 min, the absorbance at 430 nm was measured using a microplate reader (PerkinElmer, EnSpire, Singapore). Using quercetin as the standard, a calibration curve (r^2 = 0.998) was used to quantify the flavonol content, which was then expressed in µg quercetin/mg dry weight by comparing it to the quercetin standard curve.

$$\text{TFoC (µg QE/mg dry weight)} = Cq \times Vol / Mass \quad \text{(Eqn. 4)}$$

Where Cq, Vol, and Mass are the concentration of quercetin established from the calibration curve (µg /mL), the volume of the extract used in the assay (mL), and the weight of the extract (mg) used in the assay, respectively.

2.7. Antioxidant Potential

The antioxidant activity of extracts (HLE, HFE and HRE) was evaluated using DPPH, ABTS, ferric reducing antioxidant power (FRAP), and phenanthroline assays compared to BHA, BHT, α-tocopherol, and ascorbic acid. Absorbance was read using a microplate reader (PerkinElmer, EnSpire, Singapore).

2.7.1. *DPPH scavenging assay*

The ability of radical scavenging was determined according to (Fatima Zohra *et al*., 2023) method, using the radical DPPH. A volume of 40 µL of varying concentrations of the samples (3.125 –200 µg/mL in a final volume of 200 µL) were incubated with 160 µL of 0.1 mM methanolic DPPH solution for 30 minutes at room temperature in the dark. Subsequently, the absorbance of the reaction mixtures was measured at 517 nm. The scavenging activity was expressed as IC_{50} values, reflecting the concentration of the sample needed to scavenge 50% of the DPPH free radicals. These values were calculated using the linear regression method from the curve of percent inhibition versus concentration.

Percentages of inhibition of the stable radical $DPPH^{\bullet}$ were determined by the following formula:

$$I = \left[\frac{A\ control - (A\ sample - A\ blank)}{A\ control}\right] \quad \text{(Eqn. 5)}$$

Where I represents the percentage inhibition; A_{sample}, A_{blank} and $A_{control}$ are the absorbance (after 30 min) of the sample (extract or standard antioxidant), blank, and control, respectively.

2.7.2. *ABTS scavenging assay*

An assessment of the radical scavenging capacity of the extracts towards ABTS cations was performed using the methodology described by Nickavar and Esbati (2012) with some modifications. Briefly, a pre-generated $ABTS^{\bullet+}$solution was employed for the assay. 40 µL of varying sample dillutions (3.125 –200 µg/mL in a final volume of 200 µL) were reacted with 160 µL of the $ABTS^{\bullet+}$solution within a 96-well microplate. The absorbance was read at 734 nm after 10 min of incubation. The scavenging activity of ABTS was expressed as the percentage of inhibition calculated using the preceding formula **(**Eqn. 5**).** Antioxidant activity was quantified as IC_{50} values.

2.7.3. *Ferric reducing antioxidant power assay (FRAP)*

The ability of the extracts to reduce ferric iron (Fe^{3+}) ions was determined using a microplate reader adaptation of the method described by Benouchenne *et al*. (2020). A volume of 10 µL of each sample at different concentrations (3.125 –200 µg/mL in a final volume of 200 µL) was mixed with 40 µL of phosphate buffer (0.2M, pH 6.6) and 50 µL of 1% potassium ferricyanide solution. The mixture was then incubated in an oven at 50°C for 20 min. After incubation, 50 µL of 10% tri-chloroacetic acid, 40 µL of distilled water, and 10 µL of 0.1% ferric chloride solution were added, and the absorbance was directly read at 700 nm. The results were calculated as $A_{0.5}$ values, defined as the concentration required for 50% reduction in absorbance, and determined from the absorbance curve plotted with different concentrations.

2.7.4. *Phenanthroline assay*

Additionally, the reducing ability of the extracts, as measured by Fe^{+2}-phenanthroline complex formation, was determined as described by Fatima Zohra *et al.* (2023). A volume of 10 µL of each sample at final concentration (3.125 –200 µg/mL in a final volume of 200 µL) were combined in a microplate well with 30 µL of 0.5% (in methanol) phenanthroline solution, 110 µL of methanol, and 50 µL of 0.2% $FeCl_3$ solution. These reaction mixtures were incubated at 30°C for 20 minutes and subsequently analysed for absorbance at 510 nm. We calculated $A_{0.5}$ values from the regression curves.

2.8. Antibacterial Activity – Broth Microdilution Method

Eight bacterial strains isolated from hospitalized patients at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal) were investigated in this study. The strains included five Gram-negative bacteria: *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Morganella morganii*. Additionally, three Gram-positive strains were assessed: *Enterococcus faecalis*, *Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus* (MRSA).

This study utilized the methodology described by Pires *et al*. (2018). The extratcs (HLE, HFE and HRE) were first of all dissolved in 5% (v/v) dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20 mg/mL for the stock solution. Then the samples were serially diluted to obtain the concentration ranges (10 to 0.03125 mg/mL). Microdilution method and the rapid piodonitrotetrazolium chloride (INT) colorimetric assay were used to determine minimum inhibitory concentration (MIC). For the determination of MBC (lowest concentration required to kill bacteria), 10 µL aliquots from wells exhibiting no colour change were plated on blood Agar (7% sheep blood) and incubated at 37°C for 24 hours. The lowest concentration with no growth on the agar plates determined the MBC. As positive controls, ampicillin, imipenem, and vancomycin were used.

2.9. Statistical Analysis

Statistical analyses were performed with the SPSS Statistics 27.0.1 programme. The data are presented as the mean values and standard deviations (SD) of the triplicate results. Data were analysed using one-way ANOVA with a significance level of $\alpha = 0.05$. Tukey's post-hoc test at a 95% confidence level was employed to identify specific groups responsible for significant differences.

3. RESULTS and DISCUSSION

3.1. Qualitative Phytochemical Screening

Initial phytochemical screening of the three hydroethanolic extracts revealed the presence of tannins, flavonoids, and terpenoids, while alkaloids were not detected (Table 1). These detected substances are well-known for their many biological actions, which include antibacterial, anti-inflammatory, and antioxidant characteristics. Similarly, no alkaloids were found in the phytochemical screening of *P. crinita* extracts by Amor et al. (2009b) even with diverse

solvents (methanol, ethyl acetate, lyophilized infusion) and a concentrated flavonoid fraction (total oligomer flavonoids).

Table 1. Qualitative screening of hydroethanolic extracts from organs of *Phlomis crinita*.

Phytoconstituents	Test	Plant's part		
		Leaves	Flowers	Rhizome
Flavonoids	Shinoda	+	+	+
	NaOH	+	+	+
Tannins	Ferric chloride	+	+	+
Terpenoids	Salkowski	+	+	+
Alkaloids	Mayer	-	-	-
	Wagner	-	-	-
	Dragendorff	-	-	-

Note: (+): Present; (-): Absent.

3.2. Analyses of Phenolic Compounds (HPLC)

Analysis of phenolic compounds in the hydroethanolic extracts of leaves, flowers, and rhizomes of the species *P. crinita* was conducted by HPLC-DAD and the identification of specific phenolic compounds relied on comparing their retention times and UV spectra to those of authentic standards (Table 2) analyzed under the same conditions.

In this study, a total of 15 distinct phenolic compounds were detected in *P. crinita* for the first time, including four flavonoids (quercetin, epicatechin, hesperidin, catechin hydrate), t-cinnamic acid, six cinnamic acid derivatives (chlorogenic acid, sinapic acid, rosmarinic acid, t-ferulic acid, caffeic acid, p-coumaric acid), and four benzoic acid derivatives (gallic acid, syringic acid, 4-hydroxy benzoic acid, 3-hydroxy benzoic acid). Notably, quercetin was found in moderate amounts in leaf and flower extracts, with concentrations of 284.98 and 208.63 mg/kg, respectively. Chlorogenic acid was found in moderate concentrations in leaf, flower, and rhizome extracts, with concentrations of 107.35, 77.665 and 42.609 mg/kg, respectively. Furthermore, certain phenolic compounds were detected in higher levels in the rhizome extract than in leaves and flowers, including sinapic acid (165.977 mg/kg), t-ferulic acid (124.13 mg/kg), and rosmarinic acid (88.259 mg/kg). All other compounds showed concentrations below 30 mg/kg. Additionally, sinapic acid, t-ferulic acid, and catechin hydrate were absent in leaves but present in flowers and rhizomes, highlighting the variability in phenolic compound distribution among different plant parts. At the tissue level, the localization of phenolic compounds is linked to their role in the plant and can be very characteristic. For instance, flavonoids are predominantly present in the epidermis of leaves (Hunt *et al*., 2021). Phenolic content and composition can vary significantly among organs and plant species. In most instances, their accumulation remains below 1% of the dry weight (Zagoskina *et al*., 2023).

Our results have revealed that the extracts contain several compounds, including derivatives of cinnamic acid and benzoic acid, which have been associated with antioxidant, anti-inflammatory, and antimicrobial effects (Garde-Cerdan *et al*., 2017). These compounds are widely used in industries such as the pharmaceutical and food industry. For example, sinapic acid and t-ferulic acid detected in the rhizome extract are considered important dietary phenolic acids and are among the most consumed by humans (Rahman *et al*., 2022). The presence of chlorogenic acid, previously reported to enhance cellular proliferation and epithelialization, key processes associated with wound healing (Sobha *et al*., 2023), aligns with the traditional medicinal use of *P. crinita* as a wound healer plant.

Additionally, other detected compounds, such as quercetin, have numerous beneficial effects on human health. This antioxidant flavonoid is believed to offer many health benefits, providing defence against ailments including lung cancer, osteoporosis, and cardiovascular problems

(Anand David *et al*., 2016). The health benefits of phenolic compounds are determined by their structure, solubility, conjugation with other phenolic compounds or other substances, and absorption, which in turn affects their metabolism (Zeb, 2020).

Table 2. HPLC-DAD profiling of phenolic compounds in hydroethanolic extracts from organs of *Phlomis crinita.*

N°	Phenolic compound	Correlation(r^2)	RT(min)	HLE	HFE	HRE
				Concentration (mg/kg DE)		
1	Quercetin #	0.99962	76.313	284.98	208.63	6.546
2	Chlorogenic Acid **	0.99970	16.239	107.35	77.665	42.609
3	Epicatechin #	0.99879	20.169	28.110	28.631	16.486
4	Rosmarinic Acid **	0.99907	70.655	20.658	7.533	88.259
5	Hesperidin #	0.99705	65.989	6.132	3.726	5.998
6	Sinapic Acid **	0.99925	37.264	N.D	13.272	165.97
7	t-Ferrulic Acid **	0.99993	37.202	N.D	9.773	124.13
8	Caffeic Acid **	0.99892	21.476	3.687	3.536	2.893
9	3-Hydroxy-Benzoic Acid *	0.99982	22.545	3.561	6.214	3.574
10	Syringic Acid *	0. 99839	22.628	1.762	2.716	1.427
11	Gallic Acid *	0. 99966	5.912	0.204	2.157	N.D
12	Catechin Hydrate #	0.99906	11.499	N.D	2.036	6.269
13	p-Coumaric Acid **	0.99982	33.597	1.577	N.D	N.D
14	4-Hydroxy-Benzoic Acid*	0.99994	17.647	1.456	1.52	0.579
15	t-Cinnamic Acid	0.99998	75.207	1.486	1.203	9.677
16	Benzoic Acid	0.99986	47.629	N.D	N.D	N.D

Note: HLE: Hydroethanolic leaf extract; HFE: Hydroethanolic flower extract; HRE: Hydroethanolic rhizome extract; #: Flavonoids; *Benzoic acid derivatives; **Cinnamic acid derivatives; Concentration: mg/kg= ppm; DE: of Dry extract; RT: Retention time of standard; N.D: Not determined.

Furthermore, previous research supports our findings. According to Merouane *et al*. (2020), phenolic acids (such as chlorogenic, rosmarinic, and benzoic acids) and flavonoids are primarily phenolic compounds in *Phlomis* species. In a recent study by Baali *et al*. (2024), a wide range of phenolic compounds were identified in *P. crinita*. These included rosmarinic, chlorogenic, and coumaric acids, along with their derivatives, as well as quercetin derivatives. Moreover, Zaabat *et al.* (2020) isolated chlorogenic acid from the aerial parts of *Phlomis bovei De Noé*. Our study is in accordance with the literature data.

3.3. Extraction Yield

As shown in Table 3, the hydroethanolic extracts obtained from the leaves, flowers, and rhizomes of *P. crinita* yielded 15.9%, 22.8%, and 5.5%, respectively. The extraction yield from medicinal plants can vary due to several internal parameters, including genotype, organ type, and age, as well as external factors such as climate, harvest timing, and storage duration. Additionally, extraction parameters such as temperature, duration, solvent choice, and solvent/feed ratio (S/F) play a crucial role to the final yield (Rostagno & Prado, 2013). For ethanol extracts, Roby *et al*. (2013) reported yields of 14% for thyme, 18% for sage, and 15% for marjoram.

Phenolic compounds have hydroxyl groups which make them more soluble in polar organic solvents (Aryal *et al*., 2019). Therefore, we selected ethanol as the solvent. However, while methanol with its higher polarity could potentially have offered greater yields, balancing the benefits, we opted for 80% ethanol as the greener alternative. According to previous literature, Merouane *et al*. (2020) found that the yield of the hydromethanolic extracts of *P. crinita* ranged from 21.66 to 26.56% (w/w) for the leaves and from 22.26 to 30.08% (w/w) for the floral parts.

Table 3. Yield of extraction and bioactive content of hydroethanolic extracts from organs of *Phlomis crinita.*

	Yield (% w/w)	Total Phenolics (µg GAE/mg DW)	Flavonoids (µg QE/mg DW)	Flavonols (µg QE/mg DW)
Leaves	15.9	168.80 ± 5.46^{a}	65.97 ± 6,38^{a}	18.89 ± 5.12^{a}
Flowers	22.8	81.64 ± 13.29^{b}	29.58 ± 1.98^{b}	6.92 ± 1.42^{b}
Rhizome	5.5	262.62 ± 16.2^{c}	71.87 ± 3.25^{a}	14. 76 ± 2.11^{ab}

Note: w/w: mg extract mass per mg sample mass; DW: dry weight of the extract; µg GAE/mg DW: expressed as µg gallic acid equivalents per mg of DW; µg QE/mg DW: expressed as µg quercetin equivalents (QE) per mg of DW; values are means ± standard deviation (n=3); a–c: indicates that values with different letters in the same column are significantly different (p<0.05).

3.4. Quantification of Total Phenolics, Flavonoids, and Flavonols

3.4.1. *Total phenolic content (TPC)*

Extracts from different plant parts exhibited significant differences (p<0.05) in TPC. The rhizome exhibited the highest levels (262.62 ± 16.2 µg GAE/mg DW), followed by the leaves with moderate levels (168.80 ± 5.46 µg GAE/mg DW). The flowers, however, showed the lowest levels (81.64 ± 13.29 µg GAE/mg DW) (Table 3).

Phenolic compounds, which include flavonoids, phenolic acids, stilbenes, coumarins, and tannins, are the most abundant and frequently found phytochemicals in the plant kingdom (Wuttisin *et al*., 2021). Several studies have examined the TPC of *P. crinita*. For instance, Merouane *et al*. (2020) reported higher TPC in hydromethanolic extracts of *P. crinita* leaves compared to flowers for three different populations which is consistent with our findings. They found a TPC of 117.96 ± 1.70 µg GAE/mg DW in hydromethanolic extract of leaves. However, there has been no previous investigation of the bioactive compounds in the rhizome. Our findings indicated that the rhizome of *P. crinita* had highest TPC levels (262.62 ± 16.2 µg GAE/mg DW), which makes it a promising source of natural compounds. In fact, Aryal *et al*. (2019) reported a comparable value of 292.65 ± 0.42 µg GAE/mg DW, putting our findings firmly within the expected range. The observed TPC differences with other reports could be because of varying concentrations of sugars, carotenoids, or ascorbic acid; it could also be because of extraction duration, geography, or extraction techniques, all of which can affect the amount of phenolics extracted (Aryal *et al*., 2019).

3.4.2. *Total flavonoid content (TFC)*

The TFCs were evaluated using the aluminium chloride spectrophotometric technique. This method relies on the unique interaction between aluminium and flavonoids (Wuttisin *et al*., 2021). Significant variations at p<0.05 in the levels of flavonoid content were observed among different parts of the plant (Table 3). The rhizome contained the highest level (71.87 ± 3.25 µg QE/mg DW) being the richest source of flavonoids, followed by the leaves (65.97 ± 6.38 µg QE/mg DW), and the flowers (29.58 ± 1.98 µg QE/mg DW).

Our findings indicated that TFCs in leaves and flowers of *P. crinita* were higher than those reported by Merouane *et al*. (2020). They measured a TFC of 42.72 ± 0.53 mg QE/g DW and 15.85 ± 0.40 mg QE/g DW in methanol extracts of leaves and flowers, respectively, from a *P. crinita* population in Medjadja. In contrast, Baali *et al*. (2024) reported a higher TFC of 82.28 ± 0.44 mg QE/g DW in the methanolic extract of flowering tops of *P. crinita*. Given their wide-ranging applications in nutraceuticals, pharmaceuticals, medicine, and cosmetics, flavonoids have deservedly attracted considerable attention. By virtue of its anti-inflammatory, anti-mutagenic, anti-carcinogenic, and free radical scavenging activities, as well as its capacity to regulate the activity of important cellular enzymes (Panche *et al*., 2016).

Flavonoid profiles, as determined by HPLC-DAD analysis, which identifies and quantifies specific flavonoid compounds, indicated a higher concentration in the leaf extract. However, TFC measurements suggested greater abundance in the rhizome extract. This discrepancy may

be due to the diverse nature of flavonoids, which include subgroups like flavonols, flavones, and isoflavones (Eljabboury *et al*., 2023). Rhizomes might contain a higher proportion of flavonoid subgroups not detected by HPLC-DAD.

3.4.3. *Total flavonol content (TFoC)*

The leaves showed significantly higher levels of TFoC (18.89 ± 5.12 µg QE/mg DW) compared to the flowers (6.92 ± 1.42 µg QE/mg DW) at a significance level of $p<0.05$. The rhizome showed high levels (14. 76 ± 2.11 µg QE/mg DW) (Table 3). Interestingly, this result aligns with the fact that flavonols are found in lower concentrations than other phenolics (El Gharras, 2009).

The concentration in HFE aligns with a prior study by Merouane *et al*. (2019), who reported a similar flavonol content of 6.22 ± 0.05 µg RE/mg in a hydromethanolic extract of *P. crinita* flowers. Interestingly, the concentration of flavonols in HLE is almost six-fold higher than that reported for hydromethanolic leaf extracts (3.39 ± 0.06 µg RE/mg DW) in the same study. It's worth noting that the consumption of flavonols is linked to several health advantages, such as antioxidant properties and decreased chances of developing vascular disease (Panche *et al*., 2016). Quercetin, kaempferol, myricetin, and fisetin are among the most studied flavonols (Panche *et al*., 2016).

3.5. Antioxidant Potential

Four distinct *in vitro* assays were employed to evaluate the antioxidant effects of leaf, flower, and rhizome hydroethanolic extracts of *P. crinita*. This included assessing their ability to scavenge free radicals like $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, potentially via electron or hydrogen donation. Additionally, Phenanthroline and FRAP assays were conducted to explore the samples' ability to reduce iron ions through electron transfer mechanisms (Szydłowska-Czerniak *et al*., 2008). The antioxidant potential of extracts was compared to that of BHA, BHT, α-tocopherol and ascorbic acid. The sample which has a lower IC_{50} or $A_{0.5}$ presented a high antioxidant activity. A summary of the results can be found in Figure 1.

DPPH assay measures the ability of antioxidants to donate an electron to a stable free radical, reflecting their free radical scavenging activity. Based on the findings reported in Figure 1.A, HLE had significant antioxidant potential, more promising than BHT with IC_{50} values of 15.46 ± 0.45 µg/mL and 22.32 ± 1.19 µg/mL, respectively. Other plant parts (HRE and HFE) showed interesting antioxidant capabilities. The observed antioxidant activity supports the established understanding of phenolic compounds as prominent antioxidant agents. Regardless of whether HLE had lower levels of TPC as compared to HRE (Table 3), it exerted superior antioxidant activity, which may be attributed the diverse range of compounds present (not limited to phenolics) that can function according to a particular mode of action (Bakhouche *et al*., 2021). In comparison to a study conducted by Merouane *et al*. (2020), which reported variation in antioxidant activity between flowers and leaves, as well as among populations of *P. crinita*, HLE demonstrates approximately 4.78-fold greater DPPH scavenging activity than the leaf hydromethanolic extract from the Ouled Benabdelkader population of *P. crinita*. Furthermore, HFE exhibits three times higher DPPH scavenging activity compared to the flower hydromethanolic extract from the Medjadja population of *P. crinita.*

ABTS assay measures antioxidant capacity by monitoring the ability of antioxidants to quench the free radical $ABTS^{+}$ via a proposed mixed mechanism of hydrogen atom transfer (HAT) and single electron transfer (SET) reactions, leading to a characteristic color change from dark green to pale green (Ilyasov *et al*., 2020). Figure 1.B. reveals the strong antioxidant potential of HLE, evidenced by its IC_{50} value (11.71 ± 0.50 µg/mL) remarkably similar to that of BHA (7.54 ± 0.69 µg/mL). Furthermore, HFE and HRE exhibited moderate antioxidant activity. This test confirms the first test that HLE has good antioxidant activity.

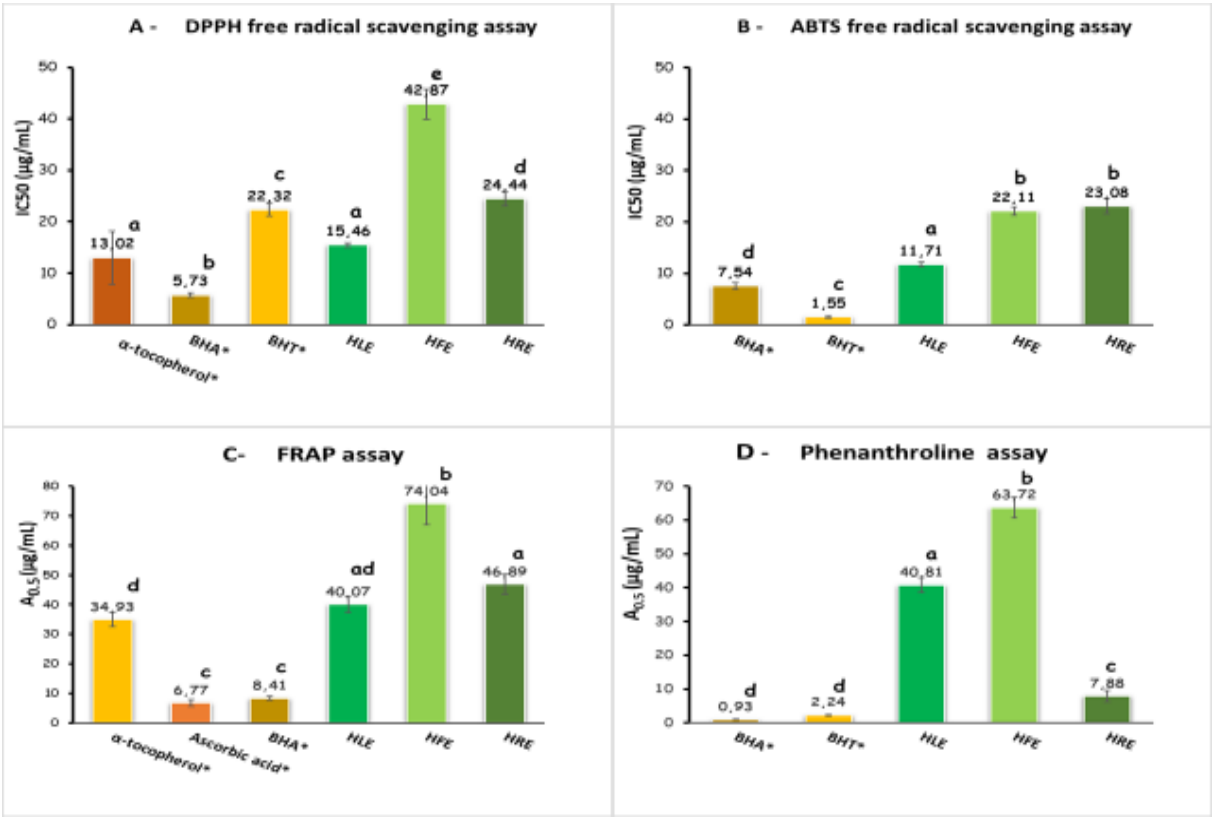

Figure 1. Antioxidant potential of hydroethanolic extracts from organs of *Phlomis crinita* using *in vitro* tests: **A:** DPPH free radical scavenging, **B**: ABTS free radical scavenging, **C**: FRAP assay, **D**: Phenanthroline assay. HLE: Hydroethanolic leaves extract; HFE: Hydroethanolic flowers extract; HRE: Hydroethanolic rhizome extract; *: Reference compound; BHT: butylatedhydroxytoluene; BHA: butylated hydroxyanisole; Values (means of three parallel measurements ± SD) followed by different lower-case letters (a-c) in the same bar graph for each test are significantly different at $p<0.05$.

The results of the FRAP assay, presented in Figure 1.C, confirm the findings of the previous tests. HLE (40.07 ± 2.82 µg/mL) and HRE (46.89 ± 3.48 µg/mL) exhibits higher reducing power $A_{0.5}$ than that of BHT (>50 µg/mL) and near to α-tocopherol (34.93 ± 2.38 µg/mL). These results may be due to the presence of electron-donating substances, since the reducing-power method follows the mode of action of electron transfer.

Phenanthroline assay measures antioxidant capacity via ferric iron (Fe^{+3}) reduction to ferrous iron (Fe^{+2}), and formation of the orange-red Fe^{2+}-phenanthroline complex (Fatima Zohra *et al*., 2023). In this assay, all extracts possess the ability to reduce iron ions, but HRE (6.95 ± 1.90 µg/mL) exhibited the highest activity, which was near to BHA (2.24 ± 0.17 µg/mL). On the other hand, HLE and HFE showed lower activity (Figure 1.D).

Overall, all extracts exhibited significant scavenging activity against DPPH and ABTS free radicals, and remarkable reducing power in the FRAP assay. Interestingly, HRE displayed the highest iron-reducing capacity in the Fe^{+2}-phenanthroline assay. These findings highlight the importance of utilizing multiple complementary assays when evaluating complex mixtures like crude extracts. This is because individual compounds within the mixture can function according to specific modes of action, and there can be potential synergistic interactions (Bakhouche *et al*., 2021). Flavonoids and phenolic acids identified in our HPLC-DAD analysis are among the compounds responsible for the antioxidant potential.

The abundance of antioxidant effects in any bioactive compound is a sign of its potential utility as a medicine or food supplement. Therefore, based on our findings, it's evident that different extracts of *P. crinita* hold great promise as sources of natural antioxidants, offering a safer alternative to potentially harmful and carcinogenic synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) (Akbaba, 2021).

3.3. Antibacterial Activity

In this study, we assessed the antibacterial activity of HLE, HFE, and HRE against clinical isolates using MIC and MBC. The values are shown in Table 4.

MIC values revealed that, all eight bacterial strains were inhibited by at least one extract type, except *Pseudomonas aeruginosa* which remained resistant at the highest tested concentration (10 mg/mL). These MIC studies revealed promising activity, particularly against Gram-positive bacteria (*E. faecalis*, *L. monocytogenes*, MRSA). Interestingly, HRE with its higher TPC compared to HLE (Table 3), exhibited strongest activity against two Gram-positive bacteria. Also, HRE exhibited the lowest MIC value (1.25 mg/mL) against *E. faecalis*, indicating the greatest susceptibility of *E. faecalis* to this extract. Even Gram-negative bacteria responded well to HRE, particularly against *M.* morganii (2.5 mg/mL) and *P. mirabilis* (5 mg/mL) compared to other extracts (10 mg/mL). This finding aligns with higher TPC of HRE suggesting a potential link between total phenolics and antibacterial activity. However, this correlation wasn't observed for *E. coli* and *L. monocytogenes* implying that other compounds besides phenolics also contribute.

Table 4. Antimicrobial potential of hydroethanolic extracts from organs of *Phlomis crinita* against clinical bacterial strains

							Positive Control					
	HLE (mg/mL)		HFE (mg/mL)		HRE (mg/mL)		Ampicillin (10mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria												
Escherichia coli	5	>10	>10	>10	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	5	>10	2.5	>10	5	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	10	>10	10	>10	2.5	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	10	>10	10	>10	5	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria												
Enterococcus faecalis	2.5	>10	5	>10	1.25	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Listeria monocytogenes	5	>10	10	>10	>10	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5
MRSA	2.5	>10	5	>10	2.5	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

Note: MIC: minimal inhibitory concentration (mg/mL), MBC: minimal bactericidal concentration (mg/mL), n.t: not tested, MRSA: Methicillin resistant *Staphylococcus aureus*.

All MBC values were higher than the highest tested concentration (10 mg/mL). While our MIC results suggest the extracts can inhibit bacterial growth at the tested concentrations, their ability to kill all bacteria remains inconclusive.

The increasing resistance of bacteria is a growing problem. This includes bacteria like *Enterococcus*, which is a leading cause of bloodstream and urinary tract infections (Chakraborty *et al*., 2015). Standard antibiotics can be very effective, but the emergence of resistant strains necessitates exploring alternative treatment options. Despite showing a higher MIC value (1.25 mg/mL) compared to standard antibiotics, our study found HRE to be effective against *Enterococcus faecalis.*

4. CONCLUSION

This study investigated the phenolic profile, bioactive content, and biological activities of *P. crinita*, a plant widely used in Algerian traditional folk remedies. The analyses revealed a

mixture of beneficial flavonoids and phenolic acids in the leaves, flowers, and rhizomes of this species, with notable quantitative variability and lower qualitative variability. The bioactive screening showed an abundance of phenolics, flavonoids, and flavonols in various parts of this medicinal herb. On the other hand, these parts exhibited strong antioxidant properties, exceeding in some cases those of synthetic antioxidants, highlighting this plant as a promising and superior source of natural antioxidants. The antibacterial properties of this species were appreciable against a panel of clinically relevant bacteria. This combination of antioxidant and antibacterial properties suggests a promising future for *P. crinita* extracts as therapeutic agents, with potential applications in the food, pharmaceutical, and cosmetic industries. This investigation significantly contributes to the existing knowledge on the secondary metabolites of *P. crinita* and the *Phlomis* genus.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Abdelhakim Chelgham: Design, Data Collection and Processing, Data interpretation, Literature review, Writing the original draft. **Abdelkader Saadi**: Supervision, Conception, Design, Critical Review. **Abdelaziz Merouane**: Conception, Design. **Chawki Bensouici:** Design, Data Processing. **Yavuz Selim Cakmak:** Design, Data Processing. **Tânia Pires:** Design and Data Collection. All authors read and approved the manuscript.

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Research Article

Phytochemical composition and antioxidant activity: Comparison of *Pentaclethra eetveldeana* (De Wild & T. Durand) leaf ethanolic extracts (Congo-Brazzaville)

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Abstract: It is important to know the intraspecific variability of the biological properties and chemical composition of plants in order to promote their better use. Thus, referring to the use of *Pentaclethra eetveldeana* leaves in a dementia traditional treatment, this study aims to highlight the antioxidant capacity and the chemical composition of the ethanolic extracts of *P. eetveldeana* leaves from four localities of Congo-Brazzaville. The β-carotene bleaching, diphenyl-picryl-hydrazyl (DPPH) radical-scavenging and molybdenum reduction methods were used to determine the antioxidant potency. Subsequently, the yields of the extractions, the phytochemical screening and the quantification of the phenolic compounds were carried out. The results revealed that the extracts of the four localities presented an antiradical and an antilipid peroxidation superior to those of ascorbic acid in DPPH and β-carotene bleaching methods. Moreover, among the extracts, those of the leaves from Boundji and Brazzaville presented the best antilipid peroxidation, antiradical and reducing activities as well as the greatest extraction yields, the greatest quantities of total polyphenols and proanthocyanidins against low levels of flavonoids. Furthermore, saponins, polyphenols, alkaloids, reducing sugars and cardiotonic glycosides were identified in all ethanolic extracts except sterols and triterpenes which were only identified in the extracts of leaves collected in Brazzaville. In addition, flavones were identified in the leaves from Owando and Makoua; flavonols in the leaves from Boundji and Brazzaville. This study showed that *P. eetveldeana* leaf ethanolic extracts exhibit antilipid peroxidation, antiradical properties and phytochemical that varied according to the region.

1. INTRODUCTION

Endemic to the Guineo-Congolese region, *Pentaclethra eetveldeana* (De Wild & T. Durand) is a plant of the Fabaceae-Mimosoideae family (Gillet, 2013). Traditionally, the bark of this plant is known to have emetic, purgative, analgesic, anthelmintic and antiparasitic properties (Bouquet, 1969). Furthermore, the leaves of this plant are used to treat dementia (Bouquet, 1969), a neurodegenerative disease in which free radicals, including the peroxyl type, are

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involved in the damage of neuronal cells, particularly by targeting lipid membranes and proteins (Błaszczyk, 2022).

Therefore, based on the traditional use of *Pentaclethra eetveldeana* (*P. eetveldeana* leaves to treat dementia (Bouquet, 1969), a previous study demonstrated the antiradical, antilipid peroxidation and phytochemical potential of aqueous extracts of these leaves collected in four different localities of the Republic of Congo (N'goka *et al*., 2023). Underlining the importance of knowing the intraspecific variability within a species for a better use of the plant, the extracts showed a higher antioxidant effect than ascorbic acid and an abundant presence of phenolic compounds, alkaloids and saponins (N'goka *et al*., 2023) witch are groups of secondary metabolites including sub-groups with antioxidant properties (Ashraf *et al*., 2013; Francenia Santos-Sánchez *et al*., 2019; Plazas *et al*., 2022) by releasing an electron or hydrogen atoms and inhibiting lipid peroxidation. Furthermore, in order to offer a better treatment in terms of efficacy and to research the highest yield of secondary metabolites, it is necessary to experiment different extraction process. This would provide more scientific data on the plant, particularly on variations in antioxidant potential and phytochemical composition. In this respect, antioxidant compounds such as polyphenols, which prevent the risk of neurodegenerative diseases (Francenia Santos-Sánchez *et al*., 2019), as well as saponins and alkaloids are well extractable by ethanol (Ato Koomson *et al*., 2018; Rajbhar *et al*., 2015) (LD50 = 6200 mg/kg), a non-toxic or very low toxic solvent with good biodegradability and low bioaccumulation potential (VWR International, 2007).

In accordance to the previous statements, this work aims to highlight the antioxidant activity and the phytochemical composition of the ethanolic extracts of *P. eetveldeana* leaves. Furthermore, to highlight any difference in the therapeutic potential and the phytochemical composition of these leaves, four localities of Congo-Brazzaville have been selected for the harvest of leaves.

2. MATERIAL and METHODS

2.1. Plant Material

Plant material was constituted by four collections of *P. eetveldeana* leaves namely from the districts of Makoua, Owando, Boundji and the department of Brazzaville in the Republic of Congo. These leaves collected (September, 2022) between 6 to 7 a.m. were dried and crushed for the study. The herbarium number: collection B. DESCOINGS n°6999, 05/06/1961.

2.2. Preparation of Extracts: Maceration

65 g of dried leaves were incubated for 72 hours in 400 mL of ethanol 90°. After filtration, the filtrate was evaporated at 60°C in an oven (Ang & Manuales, 2022; Mbengui *et al*., 2013). Thus, the ethanolic dry extract was obtained. Finally, the dry extract was weighed and the formula below was used to calculate yields. $M_{extract}$ represent the mass of dry ethanolic extract while M_{leaves} represent the mass of dry crushed leaves.

$$\text{Yield (\%)} = (M_{extract}/ M_{leaves}) \times 100$$

2.3. Phytochemical Screening

2.3.1. *Polyphenols*

The mixture of 2 mL of the extract with few drops of $FeCl_3$ give a bluish black color (N'goka *et al*., 2023).

2.3.2. *Gallic tannins*

The extract (2 mL) mixed with few drops of $FeCl_3$ (5%) give a green color for gallic tannins or a brown color for pseudo tannins (N'goka *et al*., 2023).

2.3.3. *Alkaloids*

An orange-colored precipitate appears in the test tube containing the extract (2 mL) and few drops of Dragendroff's reagent (N'goka *et al*., 2023).

2.3.4. *Flavonoids*

In the test tube containing extract (2 mL), the subsequent addition of hydrochloric alcohol (HCl/ethanol, 50:50, v/v) then magnesium shavings leading to a red color means the presence of flavonols and an orange color is for the presence flavones (N'goka *et al*., 2023).

2.3.5. *Saponins*

The saponins presence is demonstrated by a persistent foam for two or three minutes later after a vigorous shaking of the test tube containing 2 mL of the extract (N'goka *et al*., 2023).

2.3.6. *Cardiotonic Glycosides*

This test consists to observe a reddish-brown color in the tube when 2 mL of chloroform and then 2 mL of sulfuric acid are added to 2 mL of extract (N'goka *et al*., 2023).

2.3.7. *Reducing sugars*

A brick red precipice in the test tube containing extract (2 mL) and 1 mL of Fehling's liquor demonstrates the reducing sugar presence (N'goka *et al*., 2023).

2.3.8. *Sterols and triterpenes*

First 2 mL of chloroform are added in the test tube containing extract and then 2mL of sulfuric acid from the sides of the test tube: when a red ring appears, the sterols presence is justified and a reddish-brown color shows the triterpenoids presence (N'goka *et al*., 2023).

2.4. Quantification of Secondary Metabolites

2.4.1. *Total polyphenols (TP)*

Based on the Folin–Ciocalteu method (Aryal *et al*., 2019), 0,25 mL of the ethanolic extract prepared at 1 mg/mL was added to 1.25 mL of the reagent Folin–Ciocalteu 10% (w/v). Then, the addition of 1 mL of Na_2CO_3 (20%). Thus, the mixture was incubated for 10 min at room temperature in the dark. After that, by using a Thermo Scientific Genesys 10S UV-VIS spectrophotometer (Waltham, Massachusetts, USA), the absorbance was measured at 765 nm. Finally, the result was expressed as gallic acid equivalents in microgram per gram of extract (µg GAE/g).

2.4.2. *Tannins (TN)*

Based on the Obame Engonga (Obame-Engonga, 2009) described method, with few modifications, in the test tube a mix was made with 1.25 mL of distilled water, 0.25 mL of ferric ammonium citrate (28%), 0.25 mL of ethanolic extract and finally 1 mL of aqueous ammonia (10%). A period of 10 min of incubation followed. After that, at 525 nm, the absorbance was obtained. Then, in microgram of tannic acid equivalent/g (µg TAE/g), results were determined.

2.4.3. *Proanthocyanidins (PR)*

In accordance with Dicko *et al*. 2005 described method, proanthocyanidins quantification were assessed by mixing 2.33 mL of a butanol hydrochloric acid solution (30%) with 0.17 mL of ethanolic extract. This mixture was then, for 2 hours at 100°C, heated followed by a cooling step. At 550 nm, the absorbances were read and finally, in microgram apple proanthocyanidins equivalent/g (µg APE/g), results were determined.

2.4.4. *Flavonoids (FL)*

With some changes, using the $AlCl_3$ assay reported by Quettier-Deleu (Quettier-Deleu *et al*., 2000), flavonoids quantity was determined in microgram of quercetin equivalent/g (µg QE/g).

This method consists in the mix of 0.5 mL of $AlCl_3$ (2 % in methanol) with 0.5 mL of extract (1 mg/mL in methanol). Then, after waiting 10 min, at 430 nm the absorbances were determined.

2.5. Antioxidant Assay

2.5.1. *DPPH scavenging assay*

Following the method reported by Abdullahi *et al*. (2018), with some changes, 10 g of 1.1-diphenyl-2-picrylhydrazyl (DPPH) powder were prepared at 50 µg/mL in methanol (100%). Then, a mix of 1 mL of ethanolic extract or reference antioxidant (12.5, 6.25, 3.125, 1.5625 and 0.78125 µg/mL in methanol) with 1 mL of DPPH solution was made followed by an incubation in the dark, for 30 min. After that, all absorbances were read at 517 nm. The negative control consisted of a mixture of 1 mL of distilled water with 1 mL of DDPH solution. The inhibition percentages of DPPH radical were calculated using the formula below.

$$\text{Inhibition (\%)} = (\text{AbsC} - \text{AbsS}/\ \text{AbsC}) \times 100$$

AbsC means the absorbance of the negative control and AbsS is the absorbance of the extract or reference antioxidant (ascorbic acid).

2.5.2. *Molybdenum assay*

In line with Abubakar *et al*. (2013), the molybdenum method for the assessment of total antioxidant capacity consisted of the following mixture: 0.3 mL of extract with 3 mL of molybdenum reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Test tubes containing the mixtures were then heated for 1 hour 30 min at 70°C and cooled at room temperature. Finally, after the measurement of the absorbances at 695 nm, the total antioxidant capacity was determined as microgram of ascorbic acid equivalent/g (µg AAE/g).

2.5.3. *β-carotene bleaching assay*

The β-carotene linoleate assay like described in a previous study was used with few changes (Ghedadba *et al*., 2014). The reagent was prepared using 500 µg of β-carotene mixed with 1 mL of chloroform, 0.05 mL of linoleic acid and 0.5 µL of tween (20%). Then, the addition of 100 mL of distilled water followed the step of evaporating chloroform at 50°C. The mixture was vigorously shaking and an emulsion was obtained. The reaction mixture was constituted by 2.5 ml of the emulsion and 0.5 mL of extract (1mg/mL) or reference antioxidant (ascorbic acid, 1 mg/mL) or distilled water (negative control). Then, the mixtures were incubated for 2 hours at 50°C for the generation of linoleic acid free radical and 48 hours later the absorbances were measured at 470 nm. Finally, using the following formula, the relative antioxidant activity (RAA) expressed in percentage was calculated.

$$\text{RAA (\%)} = (\text{AbsS}_{(48h)}/\ \text{AbsP}_{(48h)}) \times 100$$

$\text{AbsS}_{(48h)}$ means the absorbance of the ethanolic extract or the absorbance of the negative control while $\text{AbsP}_{(48h)}$ represents the absorbance of the positive control (ascorbic acid).

2.6. Statistical Analysis

All results were analyzed using Microsoft Office Excel 2019. They were expressed as mean ± standard deviation (*SD*) and significance difference was evaluated at p =0.05. Then, using a regression analysis, the concentration of ethanolic extract that inhibit 50 % of the DPPH radicals (IC_{50}) were calculated for the data obtained from DPPH assay. Finally, to assess the link between the antioxidant activity and phytochemicals, a correlation analysis was done.

3. RESULTS

In the following line, the ethanolic extract of *P. eetveldeana* leaves from Boundji, Brazzaville, Makoua and Owando are respectively eetBO, eetBR, eetMA and eetOW.

3.1. Extraction Yields

It was found that the ethanolic extract of the leaves from Brazzaville presented the greatest yield like reported in Table 1. Among the others yields, that of the leaves from Boundji showed the highest value while the lowest value is shown by the ethanolic extract of the leaves from Owando.

Table 1. Yields of the ethanolic extracts of *P. eetveldeana* leaves.

Ethanolic extracts	Dry extracts mass (g)	Yields (%)
eetMA	2.45	3.76
eetBO	3.58	5.50
eetBR	5.04	7.75
eetOW	1.94	2.98

3.1. Phytochemical Composition

The Table 2 reports the presence of alkaloids, saponins, polyphenols, flavonoids, tannins, cardiotonic glycosides and reducing sugars in all ethanolic extracts while sterols and triterpenes were only found in the ethanolic extract of the leaves from Brazzaville.

Table 2. Metabolites identified in the ethanolic extracts of *P. eetveldeana* leaves.

Extracts	Alkaloids	Saponins	Polyphenols	Flavonoids	Tannins	ST	CG	RS
eetMA	+++	+	+++	+ (a)	++	-	+	++
eetBO	+++	+	+++	+ (b)	+++	-	+	+++
eetBR	+++	+	+++	+ (b)	+++	++	++	+++
eetOW	+	+	+++	+ (a)	++	-	+	+

Very abundant: +++, Abundant: ++, less abundant: +, not detected: -. ST: sterols and triterpenes, CG: cardiotonic glycosides, RS: reducing sugars. (a): flavones, (b): flavonols.

3.2. Phenolic Compounds Content

The Figure 1 shows the quantities of phenolic compounds from the ethanolic extracts of *P. eetveldeana* leaves. It was found that eetBO (TP: 1373.05 ± 10.35 µg GAE/g, PR: 1663.22 ± 42.50 µg APE/g), eetBR (TN: 744.66 ± 16.44 µg TAE/g) and eetOW (FL: 146.86 ± 4.31 µg QE/g) possess respectively the greatest quantity of total polyphenols, proanthocyanidins, tannins and flavonoids followed respectively by eetMA (TP: 1096.11 ± 8.42 µg GAE/g), eetBR (PR: 886.00 ± 11.54 µg APE/g), eetOW (TN: 512.07 ± 31.03 µg TAE/g) and eetMA (FL: 112.17 ± 2.03 µg QE/g)). Furthermore, the lowest quantities of total polyphenols (642.50 ± 26.78 µg GAE/g), proanthocyanidins (576.00 ± 28.03 µg APE/g), tannins (169.48 ± 20.55 µg TAE/g) and flavonoids (32.07 ± 2.62 µg QE/g) were found to be those of eetOW (TP and PR), eetBO (TN) and eetBR (FL).

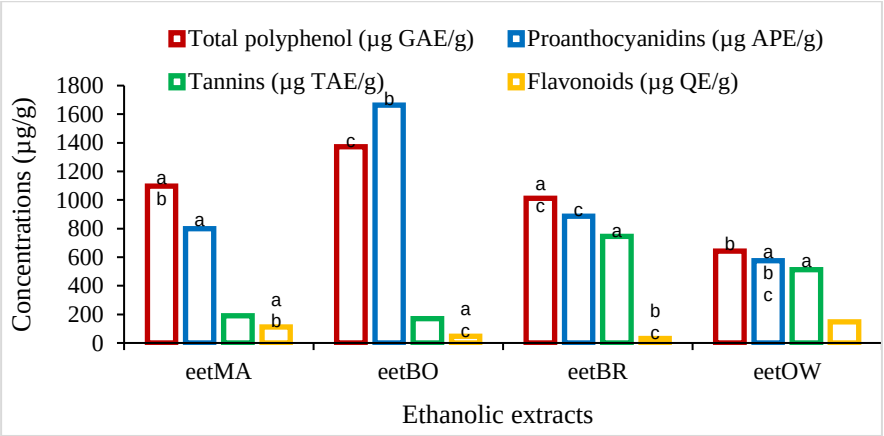

Figure 1. Phenolic contents of the ethanolic extracts of *P. eetveldeana* leaves. For each phenolic compound, the same letter means a significant difference (p=0.05).

3.3. Antioxidant Activity

The Table 3 reveals that the ethanolic extracts of *P. eetveldeana* leaves possess antioxidant activity in all methods tested. As shown below, in the case of DPPH (lowest IC_{50} represents greatest scavenging activity) and molybdenum assays, eetBR followed by eetBO exhibited the greatest scavenging power and total antioxidant capacity while concerning β-carotene bleaching assay, it was found that eetBR followed by eetMA exhibited the greatest relative antioxidant activity. Moreover, eetOW showed the lowest scavenging power, the lowest total antioxidant capacity and also the lowest relative antioxidant activity. Finally, both in DPPH (except eetOW) and β-carotene assays, the ethanolic extracts of *P. eetveldeana* leaves showed the greater activities than ascorbic acid.

Table 3. Antioxidant activity of *P. eetveldeana* leaf ethanolic extracts.

Extracts and reference	DPPH IC_{50} (µg/mL)	Total antioxidant capacity (µg EAA/g)*	Relative antioxidant activity (%)
eetMA	3.47 ± 0.20 $^{a, b}$	206.62 ± 19.67 ^{a}	179.67 ± 2.85 $^{a, e}$
eetBO	0.35 ± 0.07 ^{a}	408.91 ± 81.02 ^{b}	165.93 ± 11.03 ^{b}
eetBR	0.25 ± 0.04 ^{b}	300.79 ± 19.36 ^{c}	223.26 ± 10.21 ^{c}
eetOW	5.72 ± 0.65	126.62 ± 1.08 $^{a, b, c}$	147.25 ± 0.95 d, ^{e}
Ascorbic acid	10.27 ± 0.27	-	100.00 ± 0.00 $^{a, b, c, d}$

In each column, the same letter means a significant difference (p=0.05).
*µg AAE/g: microgram of ascorbic acid per gram of dry extract.

3.4. Correlation Analysis

Table 4 shows the results of the correlation analysis. With correlation coefficients in blue, a strong positive correlation was observed between total polyphenols and proanthocyanidins; total antioxidant capacity, total polyphenols and proanthocyanidins; DPPH scavenging activity and flavonoids.

Table 4. Correlation coefficients.

	TP	PR	Tannins	Flavonoids
PR	0.890079	1		
Tannins	-0.57122	-0.50412	1	
Flavonoids	-0.69985	-0.6656	-0.18231	1
DPPH	-0.78658	-0.72814	-0.05555	0.991331
TAC	0.894808	0.934102	-0.23841	-0.88735
RAA	0.241945	-0.00836	0.544111	-0.71607

PR: proanthocyanidins, TP: total polyphenol, Blue: positive correlation, Red: negative correlation, Clear blue or red: weak correlation.

Furthermore, with correlation coefficients in red, a negative correlation was observed between DPPH scavenging activity and total polyphenols; DPPH scavenging activity and proanthocyanidins; total antioxidant capacity and flavonoids; relative antioxidant activity and flavonoids

4. DISCUSSION

The results showed a variability in yields and phenolic compound levels of ethanolic extracts of *P. eetveldeana* leaves from one collection region to another, as did the results for aqueous extracts of the same leaves (N'goka *et al*., 2023). However, the rates recorded with aqueous extracts are higher (N'goka *et al*., 2023) than those obtained in the present study. The variation in the quantities of secondary metabolites accumulated could be justified by the difference in soils depending on where the leaves were collected, as well as by the age of the leaves (Li *et al*., 2016; Vázquez-León *et al*., 2017). Alternatively, the availability of carbohydrates or nutrients could be responsible for the variability in phenolic compound quantities and yields (Dar *et al*., 2016; Jaafar *et al*., 2012). The solvent used also has an influence, as compounds such as polyphenols and alkaloids are more soluble in ethanol and study carried out by Mbengui and al showed the greater yield of ethanolic extract compared to aqueous extract (Mbengui *et al*., 2013). Moreover, in agreement with our study, Tine *et al.* (2019) showed a variation in phenolic compound content in *Combretum micranthum* leaves from three localities.

For the phytochemical screening, phenolic compounds including tannins and flavonoids, alkaloids, saponins, reducing sugars and cardiotonic glycosides identified in all ethanolic extracts and sterols and triterpenes only present in the extract of leaves collected in Brazzaville show that these results are similar to those obtained with aqueous extracts (N'goka *et al*., 2023) of *P. eetveldeana* leaves collected in the same localities. The presence of these secondary metabolites is justified by the fact that they are soluble in ethanol. In agreement with our results, Dhayalan *et al*. (2018) also identified phenolic compounds, alkaloids, saponins and cardiotonic glycosides in the ethanolic extracts of *Spathiphyllum cannifolium* (Dryand ex Syns) leaves. In addition, the presence of sterols and triterpenes only in the leaves collected in Brazzaville could be explained by a polymorphism within the species or a herbivore-induced change that activates plant's defense system (Moore *et al*., 2014) as well as by the diversity of synthetic pathways depending on cell type or growth locality (Patra *et al*., 2013). In relation with our study, it was shown that the same species of lettuce had two dfferent metabolic strategies in terms of the type of metabolites produced (Corrado *et al*., 2021).

Finally, with regard to antioxidant potential, the free radical scavenging and the antilipid peroxidation effects of ethanolic extracts are superior to those of ascorbic acid and aqueous extracts of the same leaves as reported in the literature (N'goka *et al*., 2023), while the total antioxidant capacities of aqueous extracts reported in the literature are superior to those of ethanolic extracts (N'goka *et al*., 2023). On the one hand, the presence of polyphenols, which are known to be better antioxidants than ascorbic acid (Sharma *et al*., 2012), could explain the fact that ethanolic extracts have better antiradical and antilipid peroxidation effects than ascorbic acid. On the other hand, the high phenolic compound content of ethanolic extracts could explain their superior effects to aqueous extracts of the same plant. Indeed, the antioxidant effect of polyphenols is proportional to the number of hydroxyl groups they may contain (Lv *et al*., 2021). Furthermore, the high phenolic and alkaloid content of ethanolic extracts, as well as the presence of saponins, could explain their antioxidant power. Flavonoids and certain types of alkaloids are well documented for their antioxidant effects, which are linked to their ability to donate an electron or hydrogen atom to stabilize reactive oxygen species, and their capacity to inhibit lipid peroxidation (Ashraf *et al*., 2013; Banjarnahor & Artanti, 2015; Francenia Santos-Sánchez *et al*., 2019; Plazas *et al*., 2022). Furthermore, the difference in antioxidant effect observed between the different ethanolic extracts could be attributed to the proportion of hydroxyl or O-CH3 groups in the phenolic compounds, alkaloids and/or saponins of each extract (Lv *et al*., 2021). The antioxidant effect would also increase with the number of hydroxyl

groups on the B ring of flavonoids (Lv *et al*., 2021). This could explain the fact that the lowest flavonoid content corresponds to the highest antiradical activity.

5. CONCLUSION

This study focused on the variability of antioxidant properties and phytochemical composition of ethanolic extracts from *P. eetveldeana* leaves, depending on the region where the leaves were harvested. All ethanolic extracts of *P. eetveldeana* have antioxidant effects through inhibition of lipid peroxidation and scavenging of free radicals. These effects vary according to the qualitative and quantitative variation in secondary metabolites in the extracts.

The primary and secondary metabolites identified in the extracts, notably phenolic compounds including tannins and flavonoids, alkaloids, saponins, cardiotonic heterosides and reducing sugars, are therefore extractable by ethanol. A homogeneous production of these metabolites was observed according to the harvesting regions considered in the present study, with the exception of sterols and triterpenes, which were only identified in leaves harvested in the Brazzaville department.

These results complement those obtained from aqueous extracts, and together provide the information needed to make better use of this plant, while opening up prospects for further research into the traditional use of *P. eetveldeana* leaves to treat dementia.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Research Article

The effect of *Crocetin* on cholesterol depletion-mediated lipid raft disruption-induced apoptosis in breast cancer cells

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Abstract: The purpose of this study was to determine the effect of lipid raft function loss due to depletion of cholesterol in the lipid raft structure of cell membrane by crocetin and Methyl β cyclodextrin (MβCD) on cell viability and lipid raft-associated gene and apoptotic gene expressions of breast cancer cell (MCF-7). For this purpose, MCF-7 cells were treated with different concentrations of MβCD and crocetin. Cell viability was evaluated by WST-1 at 24 and 48 hours. The mRNA expressions of caveolin 1, LRP 6, survivin, Bcl2, Bax, and Caspase3 were assessed in the MβCD-treated group; crocetin-treated group; mixed-treated group MβCD+ crocetin MCF-7 cells by reverse transcription polymerase chain reaction at 24 h exposure. Cell viability indicated that all concentrations of MβCD decreased the viability of MCF-7 cells compared with control; reduction in cell viability was greatest with 1 mM. Additionally, exposure to all crocetin concentrations significantly reduced the cell viability of MCF-7 in a time-dependent manner. There was statistically significant down-regulation of caveolin 1, LRP-6, survivin, Bcl2 in response to MβCD, and crocetin at 24 h but Bax ve caspase 3 expressions were increased compared to control at 24h. These results indicated that crocetin application to MCF-7 in addition to MβCD regulated mRNA expression of lipid raft-associated genes and apoptotic genes. These findings suggest that crocetin affects MCF-7 function via cholesterol depletion-related deterioration in the lipid raft structure, which is critical for the induction of apoptosis in MCF-7 cells.

1. INTRODUCTION

Breast cancer, primarily due to its heterogeneous cell diversity (Yeo *et al*., 2017), is a complex disease that encompasses various clinical presentations, diagnoses, and treatment approaches (Badana *et al*., 2018). Lacking specific target markers for diagnosis, treatment, and clinical monitoring, breast cancer presents a challenge in the medical field. When compared to healthy cells, many cancer cells including breast cancer cells show an enhanced absorption of glucose. The metabolization of this acquired glucose occurs through both anabolic and catabolic pathways, leading to the excessive production of precursor molecules, heightened glutamine levels, altered lipid metabolism, and a highly proliferative and acidic microenvironment. This complexity contributes to the diverse clinical manifestations observed in breast cancer and other cancer types (Hanahan & Weinberg, 2011; Mathupala *et al*., 2006; Murai, 2015). It has been established that lipid rafts, characterized by a high content of cholesterol, within the cell

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membrane serve as pivotal signaling entities in various cancer types, notably breast cancer. These lipid rafts are implicated in fostering an augmented accumulation of cholesterol within malignant tissues as opposed to their healthy counterparts. This phenomenon is thought to have a significant impact on the initiation and progression of tumors(Hirsch *et al*., 2010; Li *et al*., 2017; Shah *et al*. 2016). Lipid rafts represent dynamic microdomains, characterized by the dense packing of fatty acid chains measuring approximately 10-20 nm in size. These microdomains are situated within the cell membrane, exhibiting an enrichment of cholesterol and sphingolipids (Li *et al*., 2017; Pike *et al*., 2006; Sezgin *et al*., 2017; Varshney *et al*., 2016).

These molecules, characterized by a highly dynamic structure, particularly within the cell membrane, intricately participate in the regulation of diverse membrane proteins through protein-protein interactions. They play a pivotal role in executing numerous cellular functions, including apoptosis and adhesion, by modulating the phosphorylation cascade through activation or inactivation mechanisms. In the context of breast cancer cells, lipid rafts establish molecular connections among dynamic membrane microdomains, contributing significantly to the process of carcinogenesis. Leveraging proteomic datasets, Shah *et al*. (2016) conducted an empirical investigation that quantitatively delineated the association between breast cancer and the abundance of lipid rafts (Shah *et al*., 2016). The study revealed a statistically significant correlation between the developmental progression of breast cancer and the intricate interplay involving cell cytoskeleton dynamics and membrane rafts. To achieve this objective, lipid raft-centric research employs the cholesterol deprivation model. Agents such as filipin, nystatin, or methyl beta cyclodextrin (MβCD) are utilized to extract cholesterol from the lipid raft structure, inducing a temporary loss of microdomain functionality. Notably, MβCD stands out as one of the most frequently employed specific agents in research endeavors aimed at effecting cholesterol deprivation from lipid raft (Zidovetzki & Levitan, 2007). Moreover, lipid rafts emerge as pivotal regulators of signaling pathways associated with diminished apoptosis, concurrent with heightened proliferation and invasion common hallmarks in the diagnosis of breast cancer cells. Notably, the fluidity and transitivity of cholesterol within the cell membrane, its intricate association with receptor functionality, ion channel activity, and its substantial presence within lipid rafts collectively underscore its profound implications in the modulation of apoptosis within cancer cells (Burger *et al*., 2000). In conjunction with these considerations, a multitude of phytochemicals, including species such as saffron (*Crocus sativus*), are presently incorporated into traditional therapeutic regimens for their discernible chemopreventive and therapeutic attributes in the realms of breast cancer diagnosis and treatment, owing to their characteristic low toxicity and heightened efficacy (Bolhassani *et al*., 2014; Patel *et al*., 2017). Saffron is a spice derived from the flower of *Crocus sativus*, renowned for its abundant carotenoid content. The flower encompasses two primary carotenoids responsible for its coloration, namely crocin and crocetin (Bathaie *et al*., 2014). Among these, crocin stands out as a crucial carotenoid of significant importance, serving as the precursor molecule to crocetin. Derived from the stigmas of the saffron plant, crocin is characterized by a short carbon chain length. This yellow-hued primary component emerges as a distinctive molecule in traditional medical applications, showcasing unique properties as an antioxidant, anti-inflammatory, and anti-tumorigenic agent (Li *et al*., 2017; Umigai *et al*., 2011). Chryssanthi *et al*. (2007) investigated the impact of four distinct *Crocus* species extracts on the viability of MCF-7 and MDA-MB-231 breast cancer cell lines through MTT analysis (Chryssanthi *et al*., 2007). The results elucidated that among the four *Crocus* species incorporated in the study, *Crocus sativus* demonstrated the most pronounced efficacy as a chemopreventive and anti-cancer agent (Chryssanthi *et al*., 2007). In our study, we chose this structure to examine the lipid raft structure and the functional effects of disruption of this structure in MCF-7 cells, especially to investigate the effects of Crocetin and Methyl β cyclodextrin. Lipid rafts are known as dynamic microdomains that play an important role in cell membrane organization and cellular signal transduction. These regions have been associated with cancer progression by providing platforms for growth factor receptors,

signaling proteins and proteins involved in apoptosis processes cancer cells including breast cancer. In particular, lipid rafts have been shown to affect signal transduction, proliferation and metastasis processes in cancer cells and it is known that disruption of these structures can directly affect the survival and proliferation potential of cancer cells. Our study aims to better understand the functional effects of lipid raft structure on MCF-7 cells and to reveal the potential effects of Methyl β cyclodextrin administration together with Crocetin on this process. Therefore, lipid raft structure was chosen as an important research topic in terms of its impact on cancer cell functions. Therefore, we propose that changes in the associated cellular functions that may result from disruption of the lipid raft structure localized in the cell membrane will play a key role in understanding the therapeutic potential of Crocetin on breast cancer cells.

In this study, the potential influence of crocetin carotenoid and methyl beta cyclodextrin (MβCD) on cell viability, along with their impact on the mRNA expression levels of lipid raft structure-associated genes (caveolin 1, LRP 6) and apoptotic genes (survivin, Bcl2, Bax, Caspase3), was investigated in MCF-7 cells (estrogen receptor-positive [+], non-metastatic, epithelial-like breast cancer cell line). The examination focused on the induction of lipid raft function loss attributable to the depletion of cholesterol within the cell membrane's inherent lipid raft structure.

2. MATERIAL and METHODS

2.1. Breast Cancer Cell Line and Culture

The MCF-7 cell line, which is an epithelial-like, non-metastatic, estrogen receptor-positive [+] cell line, was employed as a breast cancer model in this investigation. The cells were defrosted and cultured in 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% glutamine in a laminar flow cabinet supplemented with Dulbecco's Modified Eagle Medium (DMEM) after being removed from their -150°C frozen storage. After that, the cells were grown in a CO2 incubator at 37°C and 5% CO2 until they attained the confluence needed for the experiment. The cell culture media was replaced every other day after their morphological development was seen under an inverted microscope.

2.2. The Preparation of Methyl Beta Cyclodextrin (MβCD) Concentrations

Methyl Beta Cyclodextrin (MβCD) (332615, Sigma Aldrich) utilized in the experiments was commercially procured. The working concentrations employed in the experiments (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM) were prepared from the master stock and administered to the cells without delay before each application.

2.3. The Preparation of Crocetin Concentrations

The saffron carotenoid utilized in the study, Crocetin (C20H24O4, molecular weight: 328.4, Biomedicals, Santa), was commercially obtained. After dissolving the compound in dimethyl sulfoxide (DMSO) and preparing primary stock solutions, the experimental groups were further diluted to concentrations of 250 µM, 500 µM, and 1000 µM, according to the experimental requirements (Mir *et al*., 2020).

2.4. The Experimental Design of Study Groups

The cell viability experiments were performed with the following groups;

A) 0, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM MβCD (24h and 48h),

B) 250, 500, 1000 µM Crocetin (24h and 48h).

The total RNA isolation experiments were performed with the following groups;

1) MCF-7 cells+ 0 mM MβCD (only) (24h)
2) MCF-7 cells + 0.1 mM MβCD (only) (24h)
3) MCF-7 cells + 0.2 mM MβCD (only) (24h)
4) MCF-7 cells + 0.3 mM MβCD (only) (24h)

5) MCF-7 cells + 0.4 mM MβCD (only) (24h)
6) MCF-7 cells + 0.5 mM MβCD (only) (24h)
7) MCF-7 cells + 1 mM MβCD (only) (24h)
8) MCF-7 cells + 500 µM Crocetin (24h)
9) MCF-7 cells+ 0 mM MβCD+ 500 µM Crocetin (24h)
10) MCF-7 cells + 0.1 mM MβCD+ 500 µM Crocetin (24h)
11) MCF-7 cells + 0.2 mM MβCD+ 500 µM Crocetin (24h)
12) MCF-7 cells + 0.3 mM MβCD+ 500 µM Crocetin (24h)
13) MCF-7 cells + 0.4 mM MβCD+ 500 µM Crocetin (24h)
14) MCF-7 cells + 0.5 mM MβCD+ 500 µM Crocetin (24h)
15) MCF-7 cells + 1 mM MβCD+ 500 µM Crocetin (24h)

2.5. Cell Viability Analysis

Within the framework of the research, the impact of Crocetin carotenoid on cell viability, specifically about methyl beta cyclodextrin (MβCD)-mediated cholesterol depletion-induced lipid raft function loss in MCF-7 breast cancer cells, was analyzed using WST-1 at 24 and 48 hours (Sarı *et al*., 2021). MCF-7 cells were subjected to several dosages of MβCD (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM) and crocetin (250, 500, 1000 µM) in the experimental setup. For every group, six wells with 5000 cells each (n = 6) were created. On the 24th hour, 200 µL of WST-1 reagent (20 µL/well) was added to the cells after MβCD and Crocetin doses were applied to the designated experimental groups. After two hours of incubation at 37°C with 5% CO2, the cell culture was automatically agitated for one minute. Then, using an ELISA-reader spectrophotometer, the 96-well cell culture plate comprising the experimental groups was read at 480 nm to calculate dose-dependent cell viability ratios. For the 48th-hour cell viability examination, the identical experimental procedures were carried out again.

2.6. mRNA Expression Experiments

2.6.1. *Total RNA isolation*

Based on the effective concentrations determined for MβCD and crocetin according to the results of cell viability experiments, the following groups were established, and total RNA isolation was conducted at 24-hour:

1. MCF-7 cells were subjected to MβCD applications at concentrations of 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM,
2. MCF-7 cells were subjected to the application of 500 µM crocetin,
3. Following the application of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM MβCD to MCF-7 cells, 500 µM crocetin was administered to each group at 6 h.

Specifically, MCF-7 cells were seeded at a density of 25,000 cells/cm² in 60 mm cell culture dishes following the specified experimental groups. After evaluating the cell morphologies using an inverted microscope the next day, MCF-7 cells were treated according to the assigned experimental groups with 500 µM of crocetin and 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM of MβCD. After 24 hours of MβCD and crocetin treatments, the cells were washed with Phosphate-Buffered Saline (PBS), and aspirated using DMEM containing 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% glutamine. Subsequently, the cells were added to 500 µL of EZ-RNA A solution, which is a part of the RNA isolation kit, for lysis. After adding 500 µL of EZ-RNA B solution—an additional part of the RNA isolation kit to the mixture, it was centrifuged for 15 minutes at 4°C at 12,000 rpm to help separate the protein-DNA-RNA phases inside the tubes. After transferring the RNA phase to a different tube and adding 500 µL of isopropanol, the RNase-DNase-free polypropylene tubes were centrifuged for 15 minutes at 12.000 rpm and 4°C to extract the RNA pellets. The extracted total RNA samples were stored at -80°C before being used in subsequent mRNA expression assays.

2.6.2. *Complementary DNA (cDNA) synthesis*

The synthesis of Complementary DNA (cDNA) was conducted under the protocol of the cDNA synthesis kit. For this purpose, from each RNA sample, an amount containing 1 µg of RNA was utilized, and the reaction volume was adjusted to 20 µL by adding 1 µL 20XRT Enzyme Mix, 10 µL 2XRT Buffer Solution, and dH2O. The temperature cycle was applied via polymerase chain reaction (PCR) equipment, which ran for 50 minutes at 95°C and 60 minutes at 37°C. Before being used in real-time polymerase chain reaction (RT-PCR) investigations, the resultant cDNA was kept at -20°C.

2.6.3. *Real-time polymerase chain reaction (RT-PCR) experiments*

The mRNA expression levels of lipid raft-associated genes of caveolin 1, LRP 6, and apoptotic genes (survivin, Bcl2, Bax, caspase 3), as well as the housekeeping gene (GAPDH) (Table 1) were measured by RT-PCR using the SYBR Green Master Mix and analyzed with Stratagene MX3000P.

Table 1. Primer sequences of human-origin lipid raft and apoptosis-associated genes, 5'-3'

Primers	Forward (5'-3')	Reverse (3'-5')
Caveolin-1	CATGTCTGGGG-GCAAATACG	GAACTTGAAATTGGCACCAGG
LRP-6	GATTATCCAGAAGGCATGGCAG	TCCCATCACCATCTTCCA
survivin	ACCACCGCATCTCTAC	TCCTCTATGGGGTCGT
Bcl-2	CCGGGAGATCGTGATGAAGT	ATCCCAGCCTCCGTTATCCT
BAX	AGTGGCAGCTGACATGTTTT	GGAGGAAGTCCAATGTCCAG
Caspase 3	TTAATAAAGGTATC	CATGGAGAACACT
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

2.7. Statistical Analysis

In the evaluation of mRNA expressions, the comparative Ct method was employed to normalize the results (Giulietti *et al*., 2001; Livak & Schmittgen, 2001; Pfaffl 2001). Subsequently, the normalized values were compared using a one-way analysis of variance (ANOVA). Increases and decreases in the expression of target genes relative to GAPDH were presented with 95% confidence intervals, and statistically, a p-value < 0.05 was considered significant. One-way analysis of variance (ANOVA) was also utilized in the analysis of cell viability results.

3. RESULTS

3.1. Cell Viability Analysis Results

WST-1 was used to measure the effects of crocetin and MβCD concentrations on the viability of MCF-7 cells after 24 and 48 hours. The obtained findings showed that at MβCD doses (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM) (p<0.05), there was a substantial, dose- and time-dependent reduction in cell viability in comparison to the control group (Figure 1A, B). Furthermore, treatment with 250 µM, 500 µM, and 1000 µM crocetin led to a substantial reduction in cell viability that was dose- and time-dependent when compared to the control group. The largest loss was noted at a dosage of 500 µM (p<0.05) (Figure 1C, D).

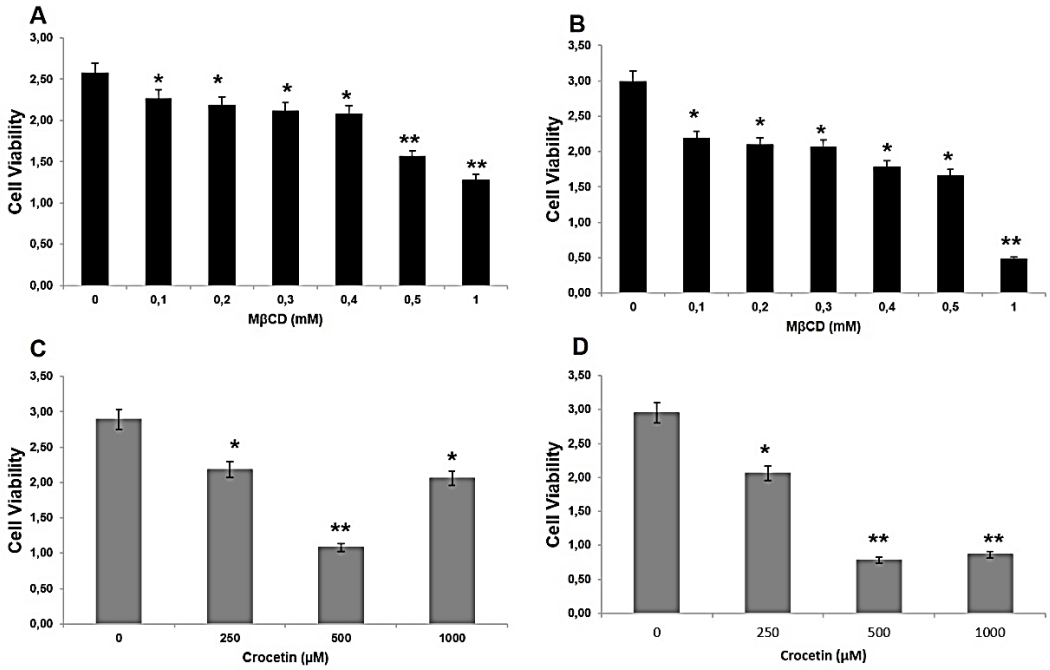

Figure 1. Cell viability analysis results from 24 hours (A) and 48 hours (B) after different concentrations of MβCD application to MCF-7 cells and 24 hours (C) and 48 hours (D) after *crocetin* application.

3.2. Lipid Raft Structure mRNA Expression Findings

Total RNA isolation was carried out at the 24-hour mark of the experiment after the cells were exposed to MβCD concentrations (0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM), crocetin concentration (500 µM), and 6 hours after the application of 500 µM crocetin following MβCD concentrations (0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM). The mRNA expression levels of caveolin 1 and LRP-6 in the lipid raft structure were next confirmed by RT-PCR assays. After applying MβCD doses to MCF-7 cells, the findings showed a dose-dependent substantial drop in caveolin 1 and LRP-6 expression levels compared to the control group, with the most significant reduction shown at concentrations of 0.5 mM and 1 mM (p<0.05) (Figure 2A, B). Additionally, it was shown that the administration of 500 µM crocetin to cells significantly decreased the levels of caveolin 1 and LRP-6 mRNA expression in comparison to the control group (p<0.05) (Figure 3). Moreover, it was shown that, six hours after MβCD application, adding 500 µM crocetin to each group significantly decreased the levels of caveolin 1 and LRP-6 mRNA expression in comparison to the control group (p<0.05). (Figure 4A, B). Additionally, the cumulative effect of adding crocetin to cells after MβCD application was found to significantly further reduce caveolin 1 and LRP-6 expressions compared to their individual applications, indicating a synergistic effect (p<0.05) (Figure 4A, B).

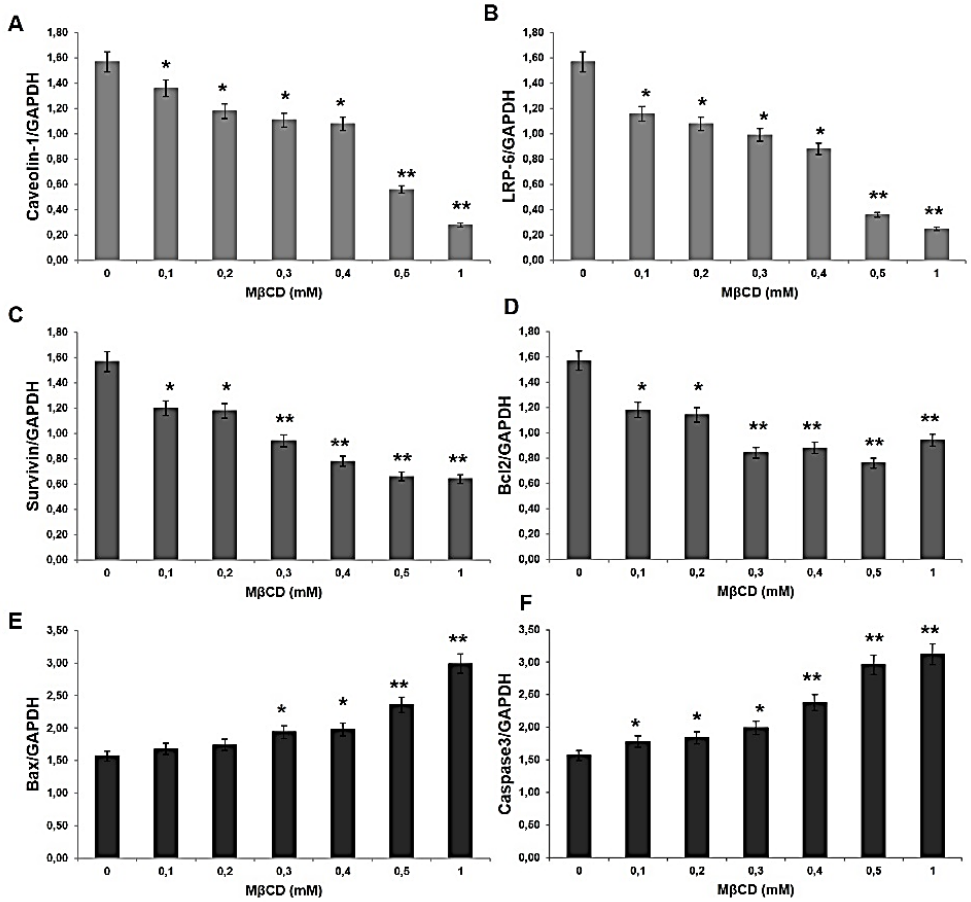

Figure 2. Caveolin 1 (A), LRP 6 (B), survivin (C), Bcl2 (D), Bax (E), caspase3 (F) mRNA expression results after only MβCD application at different concentrations in MCF-7 cells.

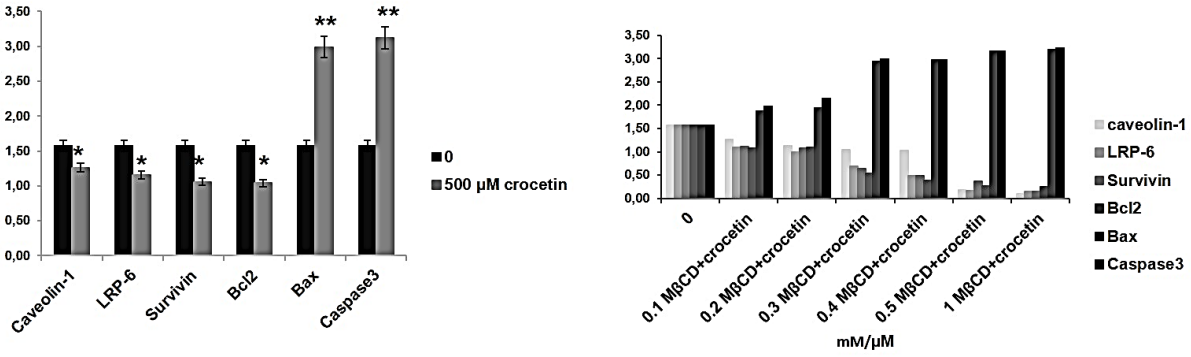

Figure 3. Caveolin 1, LRP 6, survivin, Bcl2, Bax, caspase3 mRNA expression results after only 500 µM *crocetin* application of MCF-7 cells.

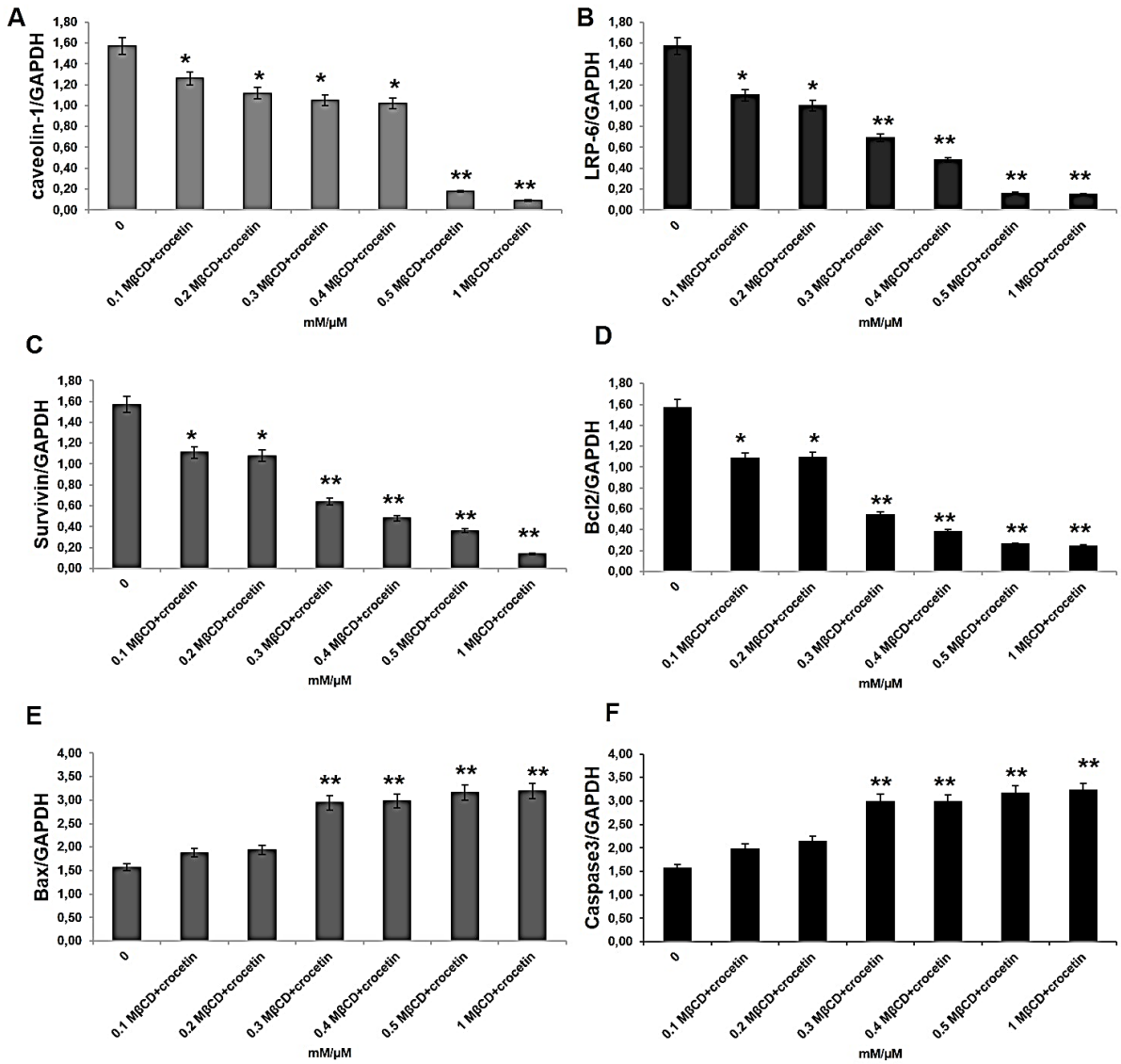

Figure 4. MCF-7 cells mRNA expression results of caveolin 1 (A), LRP 6 (B), survivin (C), Bcl2 (D), Bax (E), caspase3 (F) after application of 500 µM *crocetin* with different concentrations of MβCD.

3.3. Apoptotic Gene Expression Findings in MCF-7 Cells following MβCD and Crocetin Application

In our experiments utilizing MCF-7 cells as a breast cancer cell model under in vitro conditions, applications of only MβCD, only crocetin, and MβCD+crocetin were performed, and RT-PCR was used to examine the mRNA expressions of the apoptotic genes survivin, Bcl2, Bax, and caspase 3. According to Figures 2C and D, the administration of only MβCD resulted in a substantial drop in Bcl-2 expression and an increase in survivin expression (p<0.05) when compared to the control group. Furthermore, it was discovered that Bax expression increased at doses of 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM and that the expression of caspase 3 increased significantly in a dose-dependent manner in comparison to the control group (p<0.05) (Figure 2 C, D, E, F). Similarly, the application of crocetin alone significantly reduced survivin and Bcl2 expressions while significantly increasing Bax and caspase 3 levels compared to the control group (p<0.05) (Figure 3). Additionally, crocetin was added to MCF-7 cells six hours after MβCD was applied, and this significantly decreased the expressions of survivin and Bcl2 in a dose-dependent manner when compared to the control group (p<0.05) (Figure 4C, D). At concentrations of 0.3 mM, 0.4 mM, 0.5 mM, and 1 mM, however, Bax and caspase 3 levels were significantly increased when compared to the control group (p<0.05) (Figure 4E, F).

Specifically, the administration of MβCD+crocetin resulted in a substantial rise in the Bax/Bcl2 ratio, indicating that it promoted apoptosis in MCF-7 cells when compared to the control group.

4. DISCUSSION and CONCLUSION

The regulation of membrane proteins in cholesterol-rich lipid rafts, which are essential structural microdomains, and variations in lipid metabolism (Hirsch *et al*., 2010), including triglycerides, phospholipids, cholesterol, and cholesterol esters in cancer cells (Luo *et al*., 2017), have a major effect on the aggressive progression of tumors through a variety of cellular pathways, including adhesion, apoptosis, and regulation. The disruption and dysfunctionality of microdomain structure through cholesterol depletion are crucial for the aggressive progression of tumors (Li *et al*., 2017; Shah *et al*., 2016). In the current study, we investigated the effects of crocetin carotenoid and MβCD on MCF-7 cell viability and expression of lipid raft structure-associated genes and apoptotic genes. The estrogen receptor-positive (+), non-metastatic, epithelial-like breast cancer cell line MCF-7 had its vitality levels examined in this study utilizing WST-1 following the application of several doses (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 1 mM) of MβCD and crocetin (250, 500, 1000 μM). The results demonstrated that both crocetin and MβCD significantly reduced cell viability in a dose- and time-dependent manner ($p<0.05$). Previous studies by Badana *et al*. (2018) demonstrated that using MTT assay, MβCD doses ranging from 0.1 to 1 mM decreased the viability of breast cancer cell lines (MDA-MB 468) as well as normal breast epithelial cells (MCF-12A) (Badana *et al*., 2018). KB cells are an oral squamous cell carcinoma cell line lipid raft structures and are a frequently used in vitro model for the study of lipid raft regulation and signaling pathways as they play critical roles in cellular functions such as signal transduction, cell-cell communication, and apoptosis. Another research team treated the human oral squamous cell line KB with 20 mM MβCD for 2 hours, testing cell viability with WST-1, and demonstrated the strong cytotoxicity of MβCD on cell viability (Onodera *et al*., 2013). Maja *et al*. (2022) investigated whether cholesterol contributes to the spread of cancer cells, whether the effects are specific to cancer cells and the underlying mechanism using MCF10A cell line series (non-tumorigenic) and MDA-MB-231 cell lines (as a model of breast cancer progression and the highly invasive). Their results demonstrated that that partial membrane cholesterol depletion specifically and reversibly decreased invasion of the malignant cell lines. Also, dorsal cholesterol-enriched domains can be endocytosed and reach the cell ventral face where they were involved in invadopodia formation and extracellular matrix degradation. In contrast, non-malignant cells showed low cell invasion, low surface cholesterol exposure and cholesterol-dependent focal adhesions (Maja *et al*., 2022). The results of our study, showing a dose-dependent reduction in MCF-7 cell viability with MβCD concentrations at both 24 and 48 hours, are important in the context of similarities with existing literature findings. Crocetin is a significant carotenoid derived from the stigmas of the saffron plant, characterized by a short carbon chain length. Known for its anti-inflammatory, anti-tumor, and antioxidant qualities, this yellow-colored main component is a special chemical used in traditional medicine (Li *et al*., 2017; Umigai *et al*., 2011). By utilizing the MTT test to treat MCF-7 and MDA-MB-231 breast cancer cell lines with extracts from four distinct *Crocus* species, Chryssanthi *et al*. (2007) examined existing dose-dependent inhibition. In their research, they identified *Crocus sativus* as the most effective species with chemopreventive and anti-cancer properties among the four *Crocus* species included in the study (Chryssanthi *et al*., 2007). The bioactive components of saffron, crocin, and crocetin molecules, were found to inhibit the proliferation of cancer cells and induce apoptotic processes in cells, as evidenced by the results of the conducted study on breast cancer (Gutheil *et al*., 2012; Zhang *et al*., 2013; Zheng *et al*., 2016). Moreover, Sajjadi *et al*. (2017) through their investigation on a breast cancer model induced in Wistar albino rats using three different doses of N-methyl-N-nitrosourea, explored the anticancer effects of crocetin and crocin active molecules through different mechanisms. According to their research, crocetin and crocin molecules both work well on breast cancer cells, however, crocetin is more potent than crocin

during the beginning and advanced phases of the disease (Sajjadi *et al*., 2017). Applying 250, 500, and 1000 µM crocetin to the estrogen receptor-positive (+), non-metastatic, epithelial-like breast cancer cell line MCF-7 as a breast cancer model showed dose- and time-dependent reductions in cell viability, corroborating the body of research showing the anticancer effects of crocetin carotenoid on cancer cell viability.

In our study, the effect of 500 µM crocetin addition to cells treated with only MβCD, only crocetin concentrations, and MβCD-applied cells was investigated on the expression of lipid raft-related genes (caveolin 1, LRP 6) and apoptotic genes (survivin, Bcl2, Bax, caspase3) in MCF-7 cells using RT-PCR. The findings showed that whereas Bax and caspase 3 were upregulated, the expression of caveolin 1, LRP 6, survivin, and Bcl2 was decreased by both MβCD and crocetin treatments. Furthermore, it was noted that the target genes' expression levels were further elevated ($p<0.05$) by the addition of crocetin after the administration of MβCD. The caveolin-1 protein is an essential member of the caveolin family that plays a vital role in regulating intracellular signals, found in the cell membrane of breast cancer cells. Research findings indicated a decrease in Caveolin-1 levels in breast cancer cells due to the disruption of lipid raft structure (Badana *et al*., 2018; Elsheikh *et al*., 2008; Raghu *et al*., 2010). LRP6, a receptor in the Wnt pathway, exhibits a lipid raft localization associated with caveolin-1. Furthermore, depending on the disruption and malfunction of the lipid raft structure in breast cancer cells, the survivin gene, an inhibitor of the apoptosis family, plays a dual role in the proliferation and apoptosis cycles (Chen *et al*., 2016). Badana *et al*. (2018) used MβCD to induce cholesterol depletion in triple-negative breast cancer cell lines MDA-MB-231, MDA-MB-468, and breast epithelial cell line MCF-12A. The mRNA expressions of caveolin-1, LRP6, survivin, Bcl2, ki67, and c-myc were found to be decreased, whereas the levels of BAX and caspase-3 were increased (Badana *et al*., 2018). The Bcl-2 family, known to regulate apoptotic signals, includes the Bax gene, which, along with the p53 gene, accelerates the cell's progression to apoptosis (Naseri *et al*., 2015). The increase in the Bax/Bcl-2 ratio within the cell indicates the initiation of the apoptotic cycle associated with an increase in caspase 3. The findings demonstrated that when given in conjunction with MβCD, crocetin carotenoid both raised the Bax/Bcl-2 ratio and improved the Bax/Bcl-2 response. Although there aren't many research findings about crocetin's use in breast cancer cells in the literature, this study, which shows how crocetin works by depriving lipid raft cholesterol to have anti-proliferative and apoptosis-supporting effects, can act as a roadmap for future research. A limitation of this study is that Caveolin-1, LRP-6, survivin, Bcl-2, BAX, and Caspase-3 study results could not be included at the protein level

Cholesterol depletion in the lipid raft structures of the breast cancer cell membrane using MβCD has been shown to affect cell viability and activate genes involved in the apoptosis pathway.Following the administration of crocetin carotenoid to MCF-7 cells, a comparable and more significant impact was seen when MβCD and crocetin were applied together. The potent anti-proliferative and anti-tumor carotenoid, crocetin, derived from the saffron plant, is believed to be a potential biological agent, especially in cancer cells, highlighting lipid raft-focused cholesterol depletion through in vitro and in vivo studies that would elucidate pharmacokinetic interactions.

The findings of this study show that crocetin carotenoid decreases cell viability and promotes the apoptosis process in MCF-7 cells by suppressing MβCD-induced lipid raft dysfunction. Lipid rafts are cholesterol-rich structures in the cell membrane and play critical roles in signal transduction and cell-cell interactions (Simons & Ikonen, 2000). Signaling pathways through lipid rafts may promote cell proliferation, metastasis, and resistance mechanisms in breast cancer (Hirsch *et al*., 2010). Given that crocetin induces apoptosis by enhancing lipid raft dysfunction, this may lead to the suppression of cholesterol-dependent proliferation and signaling pathways of breast cancer cells. This feature may support the evaluation of crocetin as a potential biological agent to inhibit the progression of breast cancer cells, particularly

through cholesterol depletion occurring in lipid raft structures. Moreover, given the role of crocetin in inhibiting lipid raft-dependent cell signaling, it may contribute to the development of novel tumor progression suppressive approaches for clinical treatment strategies.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). The study was approved by the Ethics Committee of the Faculty of Medicine (2021/112) of Niğde Ömer Halisdemir University.The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Niğde Ömer Halisdemir University, 2021-112.

Authorship Contribution Statement

Şerife Buket Bozkurt Polat: Concept-Design-Supervision-Materials- Data Collection or Processing -Analysis or Interpretation- Literature Search-Writing **Esma Ozmen**: Materials-Data Collection or Processing-Analysis or Interpretation-Writing.

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Research Article

Essential oil profile and biological activity of the paleoendemic species *Salvia dorystaechas* B.T. Drew

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Abstract: *Salvia dorystaechas* B.T. Drew (syn. *Dorystaechas hastata* Boiss. & Heldr. ex Benth.) is an endemic plant native to Antalya and its surroundings, known as "Dağ çayı" and "Çalba Çayı". In the present study, *n*-hexane, ethyl acetate, ethanol 70% extracts and essential oil were obtained from the aerial parts of *S. dorystaechas.* The oil was obtained by the hydrodistillation method. The chemical composition of oil was determined by GC/FID and GC/MS analysis. Twenty-seven compounds were identified representing 99% of the oil. 1,8-cineole (26.4%), myrcene (19.2%) and α-pinene (10.1%) were determined as the main components. The extracts of the plant were screened for antioxidant activity by using a DPPH• free radical scavenging assay. The ordering of extracts in terms of antioxidant activity from highest to lowest was ethyl acetate, ethanol 70% and *n*-hexane. The extracts and hydrodistilled oil of *S. dorystaechas* were evaluated for their antimicrobial activity using standard broth microdilution protocols. The ethyl acetate extract exhibited the highest antibacterial and anticandidal activities against *S. aureus, S. typhimurium, C. utilis,* and *C. tropicalis*, with a minimum inhibitory concentration (MIC) of 62.5 µg/mL. The essential oil and the ethanolic extract demonstrated moderate to weak inhibitory effects (62,5 to >2000 µg/mL, MIC) against tested microorganisms. *S. dorystaechas* extracts demonstrate strong antimicrobial properties against various pathogens, suggesting potential use as a natural antibiotic, especially in light of increasing antibiotic resistance. Furthermore, the plant's aromatic components may be beneficial in aromatherapy. *S. dorystaechas* presents a promising candidate for natural therapeutic interventions, warranting further investigation into its pharmacological benefits.

1. INTRODUCTION

Türkiye is a country rich in plant diversity. The reason is that Türkiye has different climatic types, geological situations, geographical locations, topographic characteristics, and soil types and Anatolia has three different plant geographies region have (Davis, 1965). The family *Lamiaceae* is very rich in iridoids, alkaloids, and aromatic substances and includes about 232 genera and 8091 species in the world (Yıldız & Aktoklu, 2010; www.bizimbitkiler.org). The family *Lamiaceae*, which includes 46 genera in Türkiye, is the third largest family in terms of size with 592 species, 193 subspecies, and 42 varieties. The endemism rate is quite high, 326

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out of 844 taxa are endemic plants (www.wfoplantlist.org). Members of the family *Lamiaceae* are used both by the public and in medicine to treat many diseases. In addition to heart, vascular, gynecological diseases, and musculoskeletal disorders; It is also widely used in eye and mouth problems (Çatak & Atalay, 2022).

Salvia is the second largest genus in terms of the number of taxa (107 taxa) in the family *Lamiaceae* (Celep & Dirmenci, 2017). *Salvia* called "Sage", which has antioxidant, antibacterial, antidiabetic, and antitumor effects and more, has calming and anti-fatigue benefits and is used in the treatment of sore throat, colds, some cancers, and heart diseases (Karadal, 2022). The genus *Salvia* comprises approximately 1,050 species worldwide, 113 of which are found in Türkiye, with 58 being endemic (www.bizimbitkiler.org; www.wfoplantlist.org). *Salvia* has been claimed to be rich in essential oils and phenolic compounds, which is associated with its use in traditional medicine, pharmaceuticals, food, and cosmetics (Afonso *et al.*, 2019). *Salvia* (sage) species have been used in traditional folk medicine since ancient times as tea, ointment, tincture or extract, as an analgesic, expectorant, carminative, sedative, antiperspirant, externally as wound healer; and as medicinal plants used in the treatment of colds, bronchitis, tuberculosis, menstrual disorders, and stomach disorders. *Salvia* species primarily contain phenolic acids, flavonoids, terpenes, and terpenoids. These secondary metabolites have been reported to exhibit diverse biological effects such as antimicrobial, antifungal, antiseptic, analgesic, antioxidant, antispasmodic, antidepressant, antimutagenic, anticholinesterase, hallucinogenic, antidiabetic, anticancer, antihypertensive, anti-inflammatory, tuberculostatic, vasodilator, hypoglycemic and insecticidal activities (Elmas & Elmas, 2021).

Dorystoechas hastata Boiss. & Heldr. ex Benth. belongs to the family Lamiaceae and locally endemic, Eastern Mediterranean element plant that grows only in and around Antalya (Hedge, 1975; Güner *et al*., 2012). *D. hastata* is known as "Dağ çayı, Çalba çayı, Devren kekiği" among the inhabitant (Özcan *et al.*, 2016). It's an aromatic herb and its tea has a sharp taste, also fresh or dried leaves are consumed in the form of tea and used by locals as a medicinal drink against colds (Karagözler *et al*., 2008). *D. hastata* has a length of 40-100 cm, with pale roots measuring 18.9-29.5 cm. It features a deeply branched, woody, and globular stem, with lanceolate-hastate leaves measuring 2.2-3.5 x 5.1-8.7 cm, covered in dense hairs. The inflorescence is a spica that ranges from 6.8 to 13 cm in length. The calyx length increases as the flower (3.48-4.23 mm) develops into fruit (4.6-7.6 mm). The corolla is white and measures 4.3-6.9 mm in length. The pollen grains are isopolar, tricolporate, and measure 60 x 100 µm. The nutlets are light brown, bright, and measure 0.6-0.9 x 1.6-2.3 mm. This plant is classified as an aromatic shrub (Yılmaz, 2006). With the new classification made in 2017, *D. hastata* was transferred to the genus *Salvia* and its new name was *Salvia dorystaechas* B.T. Drew (Hedge, 1975; Drew *et al*., 2017).

This study aims to determine the chemical composition of essential oil and the antioxidant and antimicrobial activity of different three extracts of *S. dorystaechas*. While previous studies have explored the properties of *S. dorystaechas*, this research provides novel insights into its chemical composition and biological activities, highlighting its potential therapeutic applications and establishing a foundation for future investigations into its medicinal value.

2. MATERIAL and METHODS

2.1. The Plant Material

S. dorystaechas (Figure 1) was collected from Kemer/Antalya in Türkiye in June 2021. The plant material was identified by Yavuz Bülent KÖSE and the specimen is preserved in the Herbarium at Anadolu University, Eskişehir, Türkiye (Voucher specimen no: ESSE 15819).

Figure 1. *S. dorystaechas* (photo by Yavuz Bülent KÖSE).

2.2. Isolation of Essential Oil

The essential oil was obtained from the dried aerial parts of the plant. A total of 80 g of the appropriately sized material was weighed, and 10 times its weight in distilled water was added. The mixture was subjected to hydrodistillation for 3 hours using a Clevenger apparatus.

2.3. GC, GC/MS Analysis and Identification of Compounds

GC analyses were performed using an Agilent 6890N GC system. FID temperature was set to 300°C and the same operational conditions were applied to a triplicate of the same column used in GC/MS analyses. Simultaneous auto-injection was employed to obtain equivalent retention times. Relative percentages of the separated compounds were calculated from the integration of the peak areas in the GC-FID chromatograms. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system (Agilent, USA; SEM Ltd., Istanbul, Turkey). Innowax FSC column (60m x 0.25mm, 0.25µm film thickness) was used with helium as carrier gas (0.8 mL/min.). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. The split ratio was adjusted to 40:1. The injector temperature was at 250°C. The interphase temperature was at 280°C. MS were taken at 70 eV. The mass range was from m/z 35 to 450.

The components of essential oils were identified by comparison of their mass spectra with those in the in-house Baser Library of Essential Oil Constituents, Adams Library (Adams, 2007) MassFinder Library (Hochmuth, 2008), Wiley GC/MS Library (McLafferty & Stauffer, 1989) and confirmed by comparison of their retention indices. These identifications were accomplished by comparison of retention times with authentic samples or by comparison of their relative retention index (RRI) to a series of n-alkanes. Alkanes were used as reference points in the calculation of relative retention indices (RRI) (Curvers *et al*., 1985). Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.4. Extract Preparation

To prepare the extracts, 70% ethanol, *n*-hexane, and ethyl acetate were utilized on the species. The plant's aerial parts were chopped into small pieces. A 20-gram portion was weighed, and 250 mL of solvent was added. The samples underwent maceration in an orbital shaker at 150 rpm at room temperature for 48 hours. After filtration through filter paper, the solvents were evaporated using a rotavapor under reduced pressure. The 70% ethanolic extract was then placed in a lyophilizer and frozen at -20°C. The solvent-free extracts were stored at +4 ºC in a refrigerator until further use.

2.5. Antioxidant Activity

The antioxidant activities of extracts obtained from the aerial parts of *S. dorystaechas* with *n*-hexane, ethyl acetate, and 70% ethanol were evaluated by the DPPH$^{\bullet}$ free radical scavenging test procedure as described by Agiel *et al*. (2024).

2.6. Antimicrobial Activity

S. dorystaechas extracts were evaluated for their antibacterial activity against *Escherichia coli* NRRL B-3008, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Serratia marcescens* NRRL B-2544, *Klebsiella pneumoniae* NCTC 9633. Anticandidal activity was also screened by using *Candida albicans* ATCC 10231 and ATCC 90028, *C. utilis* NRRL Y- 900, *C. tropicalis* ATCC 750, and *C. parapsilosis* ATCC 22019. Anticandidal and antibacterial tests were performed according to partly modified CLSI M27-A2 and M7-A7 reference protocols. Unlike the protocols, the initial solution of the essential oil and the extracts were prepared at the concentrations of 8 mg/mL in sterile DMSO (Dimethyl sulfoxide) where the standard agents were prepared following CLSI methods. Chloramphenicol (Sigma) was used as an antibacterial while Ketoconazole (Sigma-Aldrich) was used as a standard antifungal agent (Clinical and Laboratory Standards Institute, 2006; NCCLS, 2002).

3. RESULTS

3.1. Essential Oil Components

As a result of the hydrodistillation process, a sharp-scented, pale yellow essential oil was obtained. The yield of the essential oil was calculated to be 1.625%. Both GC and GC/MS were used to analyze the essential oil. Twenty-seven compounds representing 99.1% of the essential oil were characterized by 1,8-cineole (26.4%), myrcene (19.2%), and α-pinene (7.5%) as major constituents. Table 1 displays the chemical components of *S. dorystaechas* essential oil as determined by GC-GC/MS analysis.

Table 1. The chemical components of *S. dorystaechas* essential oil.

RRI	Compounds	%	IM
1032	**α-Pinene**	**10.1**	t_R, MS
1076	Camphene	3.8	t_R, MS
1118	β-Pinene	5.8	t_R, MS
1132	Sabinene	tr	t_R, MS
1159	δ-3-Carene	3.5	t_R, MS
1174	**Myrcene**	**19.2**	t_R, MS
1188	α-Terpinene	0.4	t_R, MS
1190	Sylvestrene	0.3	MS
1203	Limonene	4.0	t_R, MS
1213	**1,8-Cineole**	**26.4**	t_R, MS
1255	γ-Terpinene	0.7	t_R, MS
1280	*p*-Cymene	0.8	t_R, MS
1290	Terpinolene	1.4	t_R, MS
1452	1-Octen-3-ol	0.2	t_R, MS
1532	Camphor	3.7	t_R, MS
1553	Linalool	0.7	t_R, MS
1591	Bornyl acetate	0.5	t_R, MS
1611	Terpinene-4-ol	1.1	t_R, MS
1612	β-Caryophyllene	3.7	t_R, MS
1684	δ- Terpineol	0.6	t_R, MS
1706	α-Terpineol	2.7	t_R, MS
1719	Borneol	3.4	t_R, MS
2008	Caryophyllene oxide	0.2	t_R, MS
2104	Guaiol	5.1	MS
2232	Bulnesol	0.5	MS
2250	α-Eudesmol	0.1	MS

2257	β-Eudesmol	0.2	MS
	Grouped compounds (%)		
	Monoterpene hydrocarbons	50.0	
	Oxygenated monoterpenes	38.6	
	Sesquiterpene hydrocarbons	3.7	
	Oxygenated sesquiterpenes	6.1	
	Others	0.7	
	Total %	**99.1**	

RRI: Relative retention indices calculated against n-alkanes; %: calculated from the FID chromatograms; tr:Trace (<0.1 %). Identification method (IM): t_R, identification based on the retention times of genuine compounds on the HP Innowax column; MS, was identified based on computer matching of the mass spectra with those of the in-house Baser Library of Essential Oil Constituents, Adams, MassFinder and Wiley libraries and comparison with literature data.

3.2. Antioxidant Activity

As a result of the DPPH$^{\bullet}$ free radical scavenging activity assay performed on extracts, the highest antioxidant activity was observed in the ethyl acetate, ethanol %70 and *n*-hexane extract, respectively (Table 2).

Table 2. The ability of extract in scavenging DPPH$^{\bullet}$ radical.

Extracts	DPPH$^{\bullet}$ IC_{50} (µg/mL)
Ethanol 70% (SA)	65.85±0.02
n-hexane (SH)	99.8 ±0.002
Ethyl acetate (SEA)	46.6 ±0.011
Gallic acid (GA)	4.8

3.3. Antimicrobial Activity

S. dorystaechas extracts were evaluated against six bacterial and five candidal reference strains. It was determined that the lowest MIC value (62,5 µg/mL) and the highest antibacterial effect against six bacterial strains were in ethyl acetate extract. *Staphylococcus aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 13311 were determined as bacterial strains with the most susceptible to ethyl acetate extract. All extracts generally showed MIC in the range of 62,5-2000 µg/mL, against bacterial strains tested. MBC values range from 125 to 2000 µg/mL. The essential oil did not show any effect at the highest dose used. It only showed an inhibitory effect against *Salmonella typhimurium* at 1000 µg/mL. *Candida* species were inhibited at concentrations in the range of 62,5-2000 µg/mL. Most active extracts against *Candida* were found as *n*-hexane and ethyl acetate, in the range of 62,5-250 µg/mL. The most susceptible strains were *C. utilis* NRRL Y-900 and *C. tropicalis* ATCC 750 (Table 3).

Table 3. Antimicrobial screening of *S. dorystaechas* herbal extracts and essential oil (MIC, µg/mL).

Microorganisms	H	EA	E	EO	S
Escherichia coli NRRL B-3008	>2000	>2000	>2000	>2000	1
Staphylococcus aureus ATCC 6538	125[1]	62.5[2]	125[3]	>2000	4
Pseudomonas aeruginosa ATCC 27853	500	500	500	>2000	32
Salmonella typhimurium ATCC 13311	125[4]	62.5[5]	125[6]	1000	1
Serratia marcescens NRRL B-2544	500	500	>2000	>2000	32
Klebsiella pneumoniae NCTC 9633	500	500	>2000	>2000	8
Candida albicans ATCC 10231	125	125	250	>2000	0.125*
C. utilis NRRL Y-900	62.5	62.5	250	250	0.06*
C. albicans ATCC 90028	250	250	500	>2000	0.06*
C. tropicalis ATCC 750	62.5	62.5	125	500	0.125*
C. parapsilosis ATCC 22019*	125	125	250	1000	0.03*

H: *n*-hexane extract, EA: Ethyl acetate extract, E: Ethanol 70% extract, EO: Essential oil, S: Chloramphenicol; *Ketoconazole; [1,2,3,4,5,6]: Minimal Bactericidal Concentrations (MBC [1]: 125 µg/mL; [2]: 500 µg/mL; [3]:2000 µg/mL; [4]: 2000µg/mL; [5]: 1000 µg/mL; [6]:2000 µg/mL)

4. DISCUSSION and CONCLUSION

4.1. Essential Oil Composition

The literature states that essential oils were extracted using hydrodistillation from the flower and leaf sections of *S. dorystaechas*. Essential oil components were analyzed by the GC and GC/MS method. The main components of floral essential oil are myrcene (19.37%), 1,8-cineol (14.30%), β-pinene (9.19%), α-pinene (8.49%) and β-caryophyllene (6.18%), while the main components of leaf essential oil myrcene (20.71%), 1,8-cineol (18.76%), β-pinene (12.51%), α-pinene (8.54%), bornyl acetate (7.28%) and terpinene-4-ol (6.19%) 11. In another study; Baser and Ozturk (1992) revealed the components of essential oils obtained from different parts (spikes, woody stems, leaves and flowering leafy) of *S. dorystaechas* by GC and GC-MS method. Essential oil compositions have been observed that vary according to the plant part obtained, the distillation method, and the place of collection. The main components were found to be 1,8-cineol, α-pinene, borneol, guaiol and camphor (Baser & Ozturk, 1992). In a study conducted in 2015, the components of essential oils obtained from the branches, leaves, and all aerial parts of *S. dorystaechas* were determined. The main components were determined as 1,8-cineol, α-pinene, borneol, guaiol and camphor. It was observed that the results of the analysis were compatible and consistent with the previous study by Baser and Ozturk (Kan *et al.*, 2015). In this study, twenty-seven compounds representing 99.1% of the essential oil were characterized by 1,8-cineole (26.4%), myrcene (19.2%) and α-pinene (7.5%) as major constituents. The analysis results were found to be compatible with previous studies. 1,8-cineole, myrcene, and α-pinene are prominent components of *S. dorystaechas* essential oil, recognized for their therapeutic potential in managing respiratory conditions, inflammatory diseases, pain relief, and anxiety reduction.

4.2. Antioxidant Activity

In this study, the antioxidant activity of *S. dorystaechas* extracts was tested using the DPPH free radical scavenging method. The highest antioxidant activity was observed in the ethyl acetate, ethanol 70%, and *n*-hexane extracts, with IC_{50} values of 46.6 µg/mL, 65.85 µg/mL, and 99.8 µg/mL, respectively. These results indicate a strong antioxidant potential, especially in the ethyl acetate extract, aligning with the findings of Karagözler *et al*. (2008) on *S. dorystaechas*. In their study, the hot water extract exhibited the highest antioxidant activity with an IC_{50} value of 6.17 ± 0.53 µg/mL, followed by diethyl ether, water, ethanol, and butylated hydroxytoluene extracts. Although the IC_{50} values for our extracts were higher, the general trend supports the significant antioxidant activity within the genus.

Additionally, Erkan *et al*. (2011) also demonstrated that petroleum ether and methanol extracts of *S. dorystaechas* exhibited potent antioxidant effects, with the petroleum ether extract showing the highest activity due to its carnosic acid and carnosol content. While our study focuses on *S. dorystaechas*, the similar behavior of ethyl acetate in both studies suggests a common antioxidant potential within these taxa. Although not all previous studies used gallic acid as a standard, they consistently demonstrate that extracts from *Salvia* species possess substantial antioxidant activity. This corroborates our findings and further indicates that *S. dorystaechas* may serve as a valuable source of natural antioxidants.

4.3. Antimicrobial Activity

In a study conducted by Balkan Bozlak *et al*. in 2020, the antimicrobial effect of eighteen oils was tested against four bacteria and three fungi. Minimum inhibitory concentration and minimum bactericidal concentrations were found. Among the eighteen oils, no effective oil was found against *A.baumanii* and *S.aureus*. *C.glabrata* showed the highest resistance to all oils including *S. dorystaechas* (Balkan Bozlak *et al.*, 2021). While there was no effective oil against *A.baumanii* and *S.aureus* among the eighteen oils in this study; In this study, *n*-hexane (125 µg/mL), ethyl acetate (62.5 µg/mL), and ethanol (125 µg/mL) extracts of *S. dorystaechas* were

found to be active against *S. aureus* ATCC 6538. In essential oils, the antibacterial effect is low (MIC>2000 µg/mL).

The major compound identified in the plant, 1,8-cineole, is known for exhibiting antimicrobial activity against various Gram-positive and Gram-negative bacteria. It particularly exerts its antibacterial effect by promoting cell membrane permeabilization and inhibiting bacterial biofilm formation. Moreover, it has been reported that 1,8-cineole reduces virulence factors and biofilm formation in bacteria by suppressing the quorum sensing (QS) mechanism (Hoch *et al*., 2023). Since hexane is an appropriate solvent for dissolving non-polar compounds, the antimicrobial activity observed in the hexane extract can be explained by the presence of non-polar components of the essential oil, such as α-pinene and myrcene.

The essential oil and extracts of *S. dorystaechas* show promising potential for applications in medicine, cosmetics, the food industry, and agriculture. Their natural, effective, and safe profiles, along with their notable antimicrobial and antioxidant activities, align with the growing consumer demand for plant-based alternatives in these areas.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Yavuz Bülent Köse took part in the data analysis and proofreading. **Gökalp İşcan** and **Mine Kürkçüoğlu** participated in the experimental work and proofreading. **Zeynep Gülcan** participated in the experimental work, preparation of the manuscript, and data analysis.

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Research Article

Chemical composition and antibacterial activity of *Azadirachta indica* seed oil from Chad

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Abstract: This study focuses on extracting oil from the seeds of the *Azadirachta indica* species (neem oil), known in Chad as mim or neem, utilizing a cold-press extraction method. It aims to characterize the oil's chemical composition through Gas Chromatography-Mass Spectrometry (GC-MS) analysis and evaluate its antibacterial efficacy using the well diffusion and microdilution techniques. The antibacterial potential was assessed against four food-borne pathogens: *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Salmonella* sp., and a soil bacterium, *Bacillus subtilis*. The results from the GC-MS analysis indicated a predominant presence of fatty alcohols, notably Stigmasta-3,5-diene (49.00%), a steroid alcohol, and Tetradecen-11-yn-1-ol (35.37%), a long-chain fatty alcohol. Additionally, lesser quantities of compounds such as (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (a diterpene) at 9.68%, Squalene (a triterpene) at 1.77%, and 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene (a sesquiterpene) at 4.19% were identified. The analysis showed that neem oil is rich in fatty alcohols and phytosterols, with lower terpenes and phenolic compounds. It showed no significant antibacterial activity against the tested bacteria. This suggests that from cold-press extraction, neem oil may not effectively combat food-borne pathogens and soil bacteria due to its fatty alcohol and phytosterol content, along with the bacteria's resistance. Increasing the concentration of crude oil in the antibacterial test could lead to positive results. The findings indicate a need for further research to isolate stronger antibacterial molecules in neem oil by separating its components, focusing on extraction methods and solvent polarity.

1. INTRODUCTION

Azadirachta indica A. Juss (called in the local Chadian language mim or neem or mimay) is a fast-growing tropical evergreen tree that belongs to the family of Meliaceae and is easily available in the Chadian areas and tropical and semitropical regions of Africa, Asia like India,

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Pakistan, and America, with various medicinal values (Gupta *et al.*, 2019; Pankaj *et al.*, 2011). The tree is often covered with delicate flowers in the early summer. The neem tree produces green drupes that turn golden yellow in June-August. Its flowers droop in panicles up to 25 cm long (Gupta *et al.*, 2019). Neem tree parts, including leaves, bark, fruit, flowers, oil, and gum, are believed to treat conditions like cancer, hypertension, heart disease, and diabetes (Islas *et al.*, 2020). The neem tree is a potential solution to prevent the Sahara Desert from spreading southward in Africa (Kumar & Navaratnam, 2013). For centuries, neem has been used to treat human diseases (Arulkumar *et al.*, 2019).

The neem tree's fruits and seeds are used to extract oil for medicinal applications (Abbas *et al.*, 2020), and are widely used in pharmaceuticals, agriculture, and other fields (Das *et al.*, 2021). Currently, researchers and communities are showing interest in various parts of the neem plant to extract benefits. However, among all the parts, the oil seems to be the most popularly used (Patel *et al.*, 2016). The seeds from neem fruits contained oil varying in the range of 25–45% oil load (Das *et al.*, 2020). Neem oil is also used as an insecticide, lubricant, and for treating diseases like diabetes and tuberculosis (Puri, 1999; Ragasa *et al.*, 1997). Neem oil is a medicinally and chemically complex lipid that has a wide range of pharmacological activities (Momchilova *et al.*, 2007; Someya *et al.*, 2018).

Gas chromatography-mass spectrometry (GC-MS) is an important technique that is now being used to identify bioactive chemicals both qualitatively and quantitatively in crude plant extracts in leaves and seeds (Muzahid *et al.*, 2023).

Few studies in Chad reported the chemical composition and the medicinal properties of neem seed oil even if neem is a well-known plant for the primary cure treating many diseases in Chad. This research study focused on the chemical composition of neem seed oil by GC-MS analysis, and the evaluation of its antibacterial activity against fourth food-borne pathogenic bacteria strains namely *Staphylococcus aureus* (*S. aureus*), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (*E. coli*), *Salmonella* sp. and against the soil bacterium *Bacillus subtilis* (*B. subtilis*).

2. MATERIAL and METHODS

2.1. Plant Material

Neem seeds were collected from N'Djamena (Chad) in February 2023. Almond (kernels) contained in the seeds of neem was obtained after hulling the grains (Figure 1). Once obtained after shelling, the almonds are stored in a hermetically sealed box for the future cold-pressure extraction of their oil.

Figure 1. a: Neem plant and fresh seeds. b: Neem seeds dried. c: almond of seeds.

2.2. Oil Extraction Method

Neem oil from seed almonds is obtained by chemical or mechanical extraction. Chemical extraction offers an oil yield of around 100%, however, the characteristics (iron, copper, and phosphorus contents) show, among other things, that neem oil from cold pressing has a satisfactory quality (Svitlana *et al.*, 2012). The best oil yields by pressing have been obtained by several authors such as (Soetaredjo *et al.*, 2008; Svitlana *et al.*, 2012) at the temperature of 30°C and 25°C respectively.

In this study, the physicochemical properties of neem almond seed and the oil extracted from it are the Mass of pressed almonds: 0.400g, Pressure temperature: 25°C. Liquid at room temperature, Progressive solidification at 16°C, Volatility (very odorous and pungent character), PH: 2, Solubility: Insoluble in water and methanol. Soluble in hexane, Density: 0.92-0.97, Saponification index: 175-205 (Figure 2).

There are many methods to extract oil from neem seed, including the mechanical method such as hydraulic press, screw press, or cold press, and the chemical methods such as solvent extraction, steam distillation, and enzyme extraction (Çakaloğlu *et al.*, 2018). In this study, we have mainly chosen the mechanical pressing extraction method due to its high oil yield even with small quantities of well-crushed almonds and also given that this type of extraction does not affect the quality of the oil obtained according to the literature (Çakaloğlu *et al.*, 2018; Nahak & Sahu, 2011; Svitlana *et al.*, 2012; Tsimidou *et al.*, 2020). According to Liauw *et al.* (2008), in their study on the quality of neem seed oïl, they found that an increase in temperature led to a decrease in iodine value but an increase in saponification, acid, and peroxide value. This suggests that higher temperatures resulted in higher neem oil yield but lower neem oil quality.

The extraction was carried out at room temperature without thermal pretreatment of the almonds. The oil was obtained by cold extraction under vacuum pressure from the oil press machine (1500W, 110V, Oil Press Professional 2019, Spain). Simply put, the pre-treated neem seeds are loaded into the feeding hopper. With the pushing action of the rotating screw shaft in the pressing chamber of the screw oil press machine, the material is pushed forward continuously. At the same time, due to the reduction of the screw pitch of the oil press, the increase of the width of the screw thread, the diameter of the root circle gradually increased (and the space of the oil press chamber decreased with the advance of the material), the material volume was compressed and a strong extrusion pressure was generated. In this way, the oil is squeezed out from the gap of the pressing cage, and the dry materials are pressed into oil cake blocks and discharged from the end of the pressing shaft automatically. The optimal extraction pressure for whole, uncrushed almonds is approximately 30.4MPa ± 4.1 (300bar). The extraction rate is 40.1% ± 1.1. The optimal height of the pressing cage is 40 mm, with a speed of around 1MPa/s and a delay time of 353s (approximately 6min). The oil obtained is pure, however a decantation was done to make at least the base even purer. This oil should be stored dry, away from heat and light. To avoid degrading the active ingredients, it is essential not to heat it above 60°C (Figure 2).

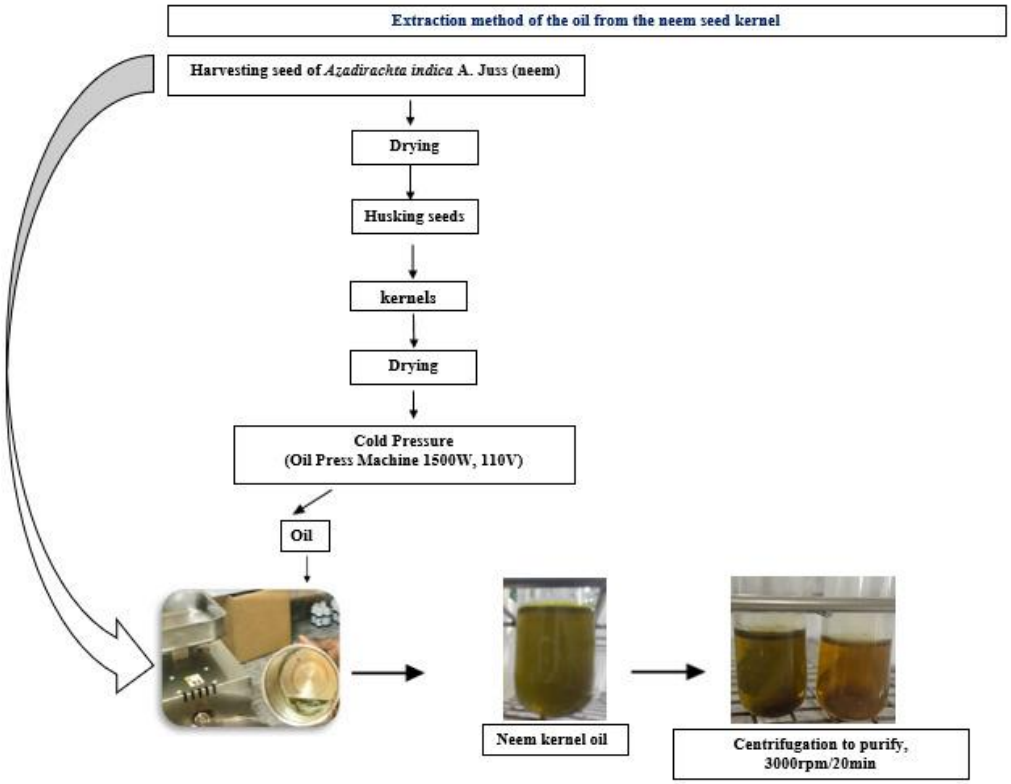


Figure 2. Process followed during the automatic cold pressure extraction of neem seed.

2.3. GC-MS Analysis

GC-MS separates chemical mixtures and identifies the components at a molecular level by heating them and carrying the gases through a column with an inert gas. The separated substances then flow into the MS (Rood, 2000; Medeiros, 2018). The analysis of the volatile fraction of the oil was carried out by a gas chromatography system coupled with mass spectroscopy type I Perkin Elmer Mass Spectrometer Clarus R SQ 8S with an auto-injector of the "AOC- 20i". The analyses are then carried out on an SLB-5ms column (30m length × 0.25mm diameter × 0.25µm film thickness). The initial temperature will be set at 50°C, then increased to 250°C as injector temperature (rate of increase: 3°C/min; holding time: 5min) (Muzahid *et al.*, 2023). The oil was solubilized in 0.5ml of n-hexane and injected after mixing with 1ml of n-hexane (split ratio: 0.5:1). The injection volume for the neem oil sample was 1µL Methylation was unnecessary as the sample was already dissolved in hexane. Hexane was used as the injection solvent to improve fraction separation, as it is a nonpolar solvent that speeds up the column process (Haleyur *et al.*, 2016). The process of pyrogenation was utilized in the GC-MS analysis. Pyrogenation, an alternative to the process of pyrolysis in GC-MS analysis, involved heating the sample to decompose and produce smaller molecules. These smaller molecules were then separated by gas chromatography and detected using mass spectrometry.

The ionization mass spectroscopic analysis was done with 70eV. Mass spectra were recorded across the range from 45*m/z* to 350*m/z* for 50.0min. The solvent cut time was 3min and the total run time was 50.0min. The bioactive compounds were identified based on retention time, MS similarity matching fragment ions generated, and an LRI filter, and the percentage of these bioactive compounds was evaluated from the total peak area (Muzahid *et al.*, 2023). Other parameters are: Linear speed: 30.0cm/s; Inlet pressure: 26.7KPa. Interface temperature: 250°C.

2.4. Antibacterial Activity

2.4.1. *Bacterial strain and growth conditions*

Five bacteria were tested: *Staphylococcus aureus* ATCC 25,923, *Methicillin-resistant Staphylococcus aureus* (MRSA), *Salmonella* sp., *Escherichia coli* K12, and *Bacillus subtilis* 6633. The bacteria are stored on an inclined agar medium (0.15) at 4°C and revived them twice

in Lysogeny Broth (LB) at 37°C for 18 to 24hours before testing. We used final inoculum concentrations of 10^6CFU/mL of bacteria as per the guidelines of the National Committee for Clinical Laboratory Standards, USA (CLSI, 2012).

2.4.2. Disc Diffusion Method

Glass cylinders were placed on a layer of Muller-Hinton agar. Lauria Bertoni medium containing 0.15% agar with bacteria. 50µL of neem almond oil was added to the cylinders. No solvent is used in order to only promote the activity of the sample. Petri dishes were incubated (37°C for 24h) and the inhibitory zone was observed (Belmehdi *et al.*, 2021; Bouhdid *et al.*, 2010; Laghmouchi *et al.*, 2018). To determine the minimum inhibitory concentration (MIC), 96-well sterile microplates were used. Initial sample 100µL of oil was diluted in wells from highest to lowest concentration, and each well received 50µL of bacterial inoculum (CLSI, 2012). Control wells had no oil sample. The microplates were then incubated for 18hours at 37°C. The MIC was determined by verifying growth with resazurin as a redox indicator. After 2hours of waiting, we found that the lowest amount of neem oil that didn't change the colour of the resazurin dye was the minimum inhibitory concentration (MIC) (Belmehdi *et al.*, 2021). The standard control antibiotic is chloramphenicol, and all tests were evaluated three times.

3. FINDINGS

3.1. GC-MS Analysis

The seed oil of neem dissolved in *n*-hexane was analyzed by GC-MS, and the results showed majority of compounds included a high quantity of long-chain fatty alcohol and phytosterol (steroid alcohol), and less quantity of terpenes (Figure 3, Table 1). Among the fatty alcohols, Stigmasta-3,5-diene (a phytosterol) was the most abundant (49.00%), followed by 13-Tetradecen-11-yn-1-ol (35.37%) (a long chain fatty alcohol).

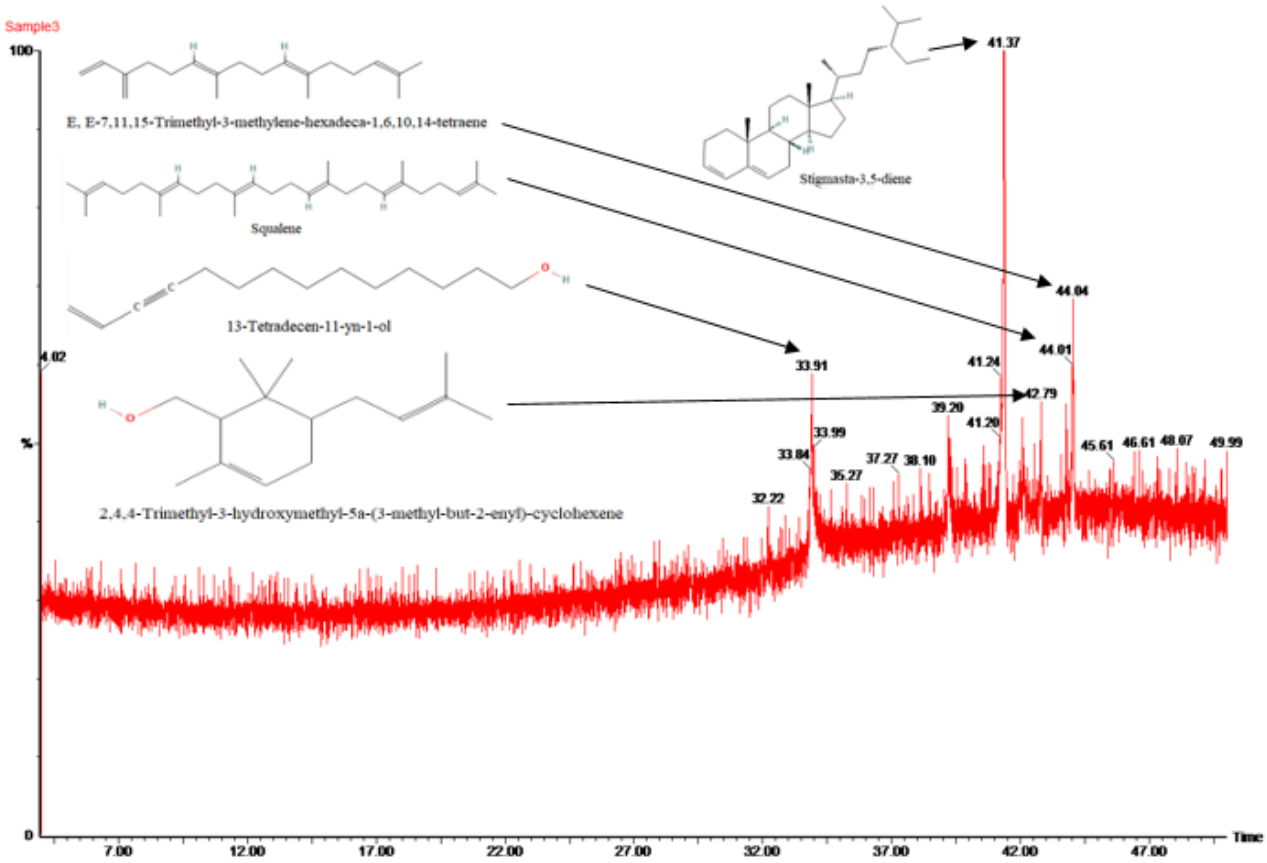


Figure 3. GC-MS chromatogram of neem seed oil.

Table 1. Showing the peak assignment of major and minor compounds detected in neem seed oil by GC-MS analysis.

Compound	RI	RT	Peak air	Area (%)	Compound	Chemical nature	Formula and Molecular weight (g/mol)	Biological Property	References of the biological property in the literature
1	-	33.91	9528973	35.37	13-Tetradecen-11-yn-1-ol	Fatty alcohol	$C_{14}H_{24}O$ (208.34)	Toxic to brine shrimp larvae	(Adedoyin *et al.*, 2013)
2	2719	41.37	13199198	49.00	Stigmasta-3,5-diene (derivate from B-Sitosterol)	Phytosterol or fatty steroid alcohol	$C_{29}H_{48}$ (396.73)	Anti-microbial	(Murniasih *et al.*, 2023)
3	-	42.79	1127420	4.19	2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene	Sesquiterpene	$C_{15}H_{26}O$ (222.37)	Anti-bacterial	(Karthik *et al.*, 2015) (Sharma *et al.*, 2018)
4	2832	44.01	475554,3	1.77	Squalene (Spinacene)	Triterpene	$C_{30}H_{50}$ (410.73)	Treats cardiovascular disease. As pesticide, as alternative for antivenom, as anticancer agent. As emollient, antioxidant, and for hydration and its antitumor activities	('Izzah Ibrahim *et al.*, 2020) (Tulashie *et al.*, 2021) (Farjaminezhad & Garoosi, 2020) (Fox *et al.*, 2023) (Huang *et al.*, 2009)
5	-	44.04	2606637	9.68	(E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (β-Springene)	Diterpene	$C_{20}H_{32}$ (272.5)	Antimicrobial	(Kamazeri *et al.*, 2012)
				Total: 100%					

The other compounds such as the diterpene (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (9.68%), the sesquiterpene 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl) cyclohexene (4.19%) and triterpene squalene (1.77%) were showed as the minority compounds present. The percentage of similarity ranged from 40.8 to 0.15%. The data from GC-MS chromatogram of neem oil n-hexane fraction displays the raised retention time peaks at 33.91, 41.19, 43.77, 44.04, and 44.35 representing respectively 13-Tetradecen-11-yn-1-ol, Stigmasta-3,5-diene, 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene, squalene, and (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (Figure 3).

3.2. Antibacterial Activity

The results of the disc diffusion method of neem seed oil showed no inhibition zone against the bacterial strains tested *S. aureus*, *MRSA*, *Salmonella* sp., *E. Coli*, and *B. subtilis* (Figure 4). In the MIC test, resazurin was completely reduced by bacterial metabolism (Figure 5).

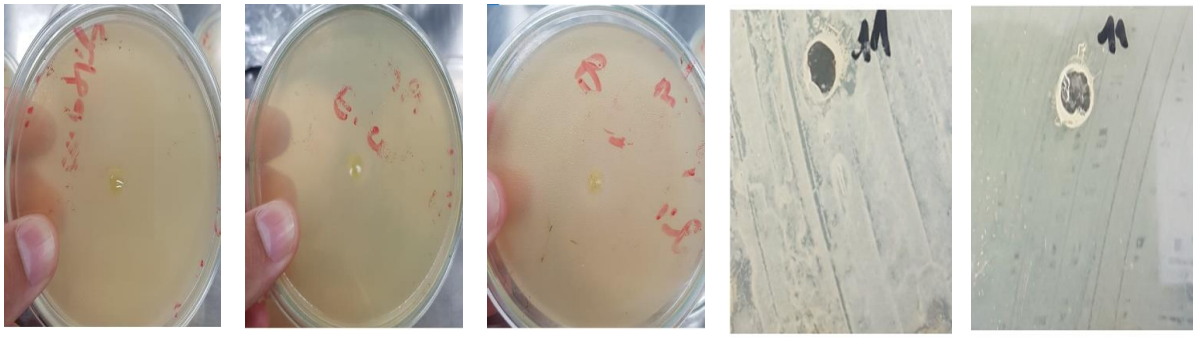


Figure 4. No antibacterial activity of neem seed oil on bacteria tested: From left to right respectively *S. aureus*, *E. coli*, *B. subtilis*, MRSA, and *Salmonella* sp.

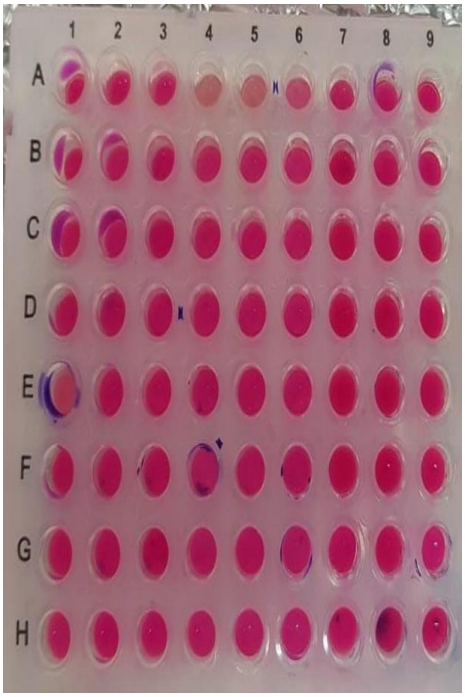


Figure 5. Resazurin dye test for determining minimum inhibitory concentration MIC (as % (v/v)) of neem seed oil. No MIC activity was revealed because resazurin was reduced here in a hot pink color.

4. DISCUSSION

This study demonstrated that neem oil obtained through pressing contains various compounds, which have been analysed through GC-MS. The mass spectra peak of the compound 13-Tetradecen-11-yn-1-ol in Figure 6 on the left is similar to peaks obtained from the National

Center for Biotechnology Information (NCBI) website data and PubChem Compound Summary for CID 543337 (National Center for Biotechnology Information (NCBI), 2024) (Figure 6, right). These peaks are shown at the m/z top peak at 55, m/z 2nd highest at 41, and m/z 3rd highest at 67 (Figure 6). The fatty acid alcohol 13-Tetradecen-11-yn-1-ol (7.83%) was identified as a major constituent of the stem essential oils from the Euphorbia heterophylla plant (Spurge Weed), extracted using the hydro distillation Clevenger method in the GC-MS analysis (Adedoyin *et al.*, 2013). The essential oil of the stem contains major compounds such as alcohols and epoxides, including 3,7,12,15-tetramethyl-2-hexadecen-1-ol, octadecanoic acid, oleic acid, linoleic acid, 1,2-epoxy-cyclododecane, cis-cis-7, 10, -hexadecadienal, 1,2-benzene dicarboxylic acid diisooctyl ester, phytol, and phthalic acid (Adedoyin *et al.*, 2013). The antimicrobial test showed moderate to low activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia* and *Candida albicans* (Adedoyin *et al.*, 2013).

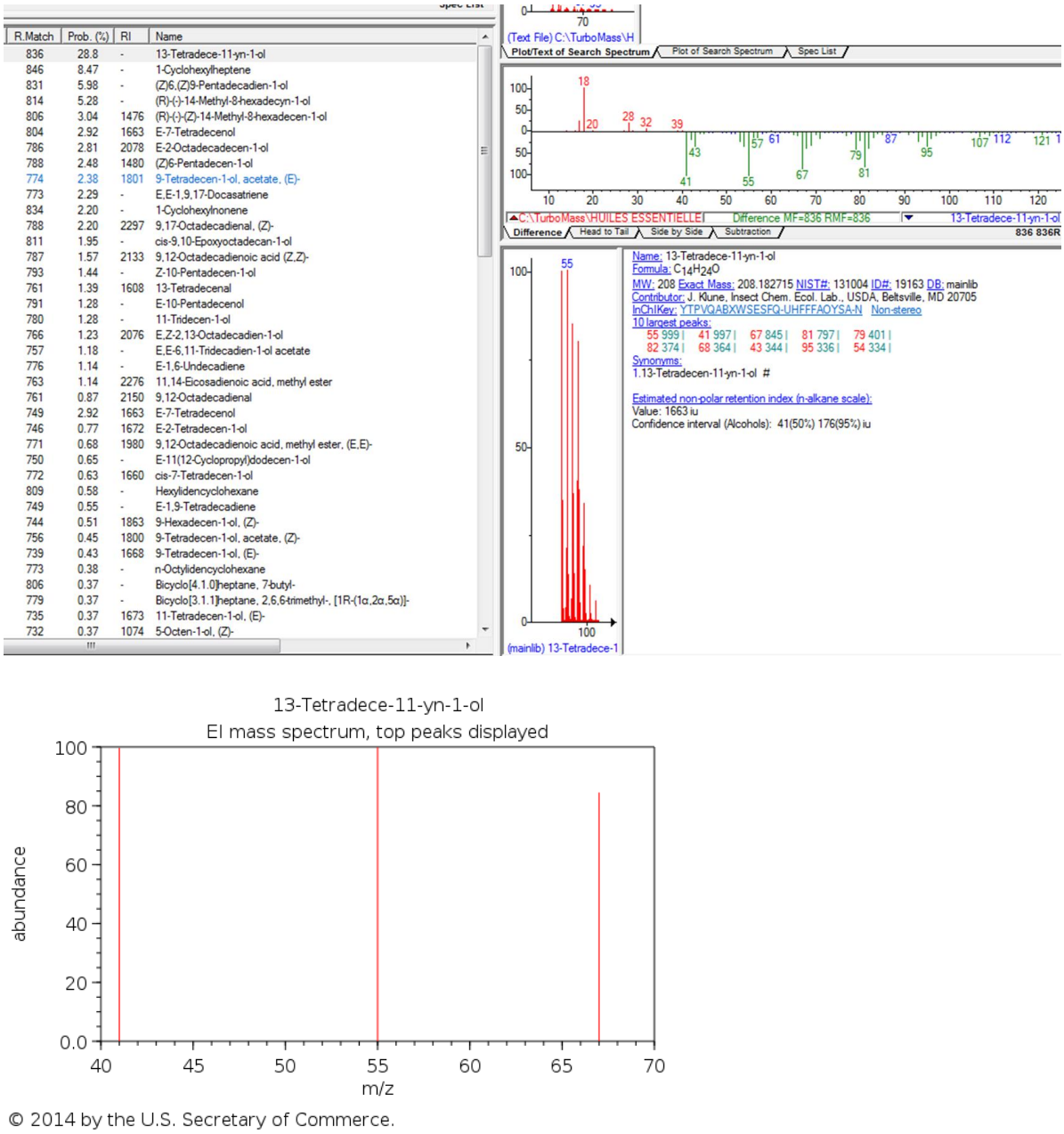


Figure 6. Mass Spectrometry GC-MS of 13-Tetradecen-11-yn-1-ol (above). The reference figure below is for the website of the National Center for Biotechnology Information website (National Center for Biotechnology Information (NCBI), 2024).

In our GC-MS analysis, the peaks of the compound Stigmasta-3,5-diene (fatty steroid alcohol) revealed in the Figure 7 in left are similar to the peaks obtained by the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024) (Figure 7, middle and the right).

The mass spectra displaying the peaks of the compound 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene are depicted in Figure 8. The molecular structure indicates that the mass spectrum reveals a peak at 32.0575 Daltons. This peak can be attributed to the bombardment of the compound, which causes the fragmentation of two methyl (CH3) molecules.

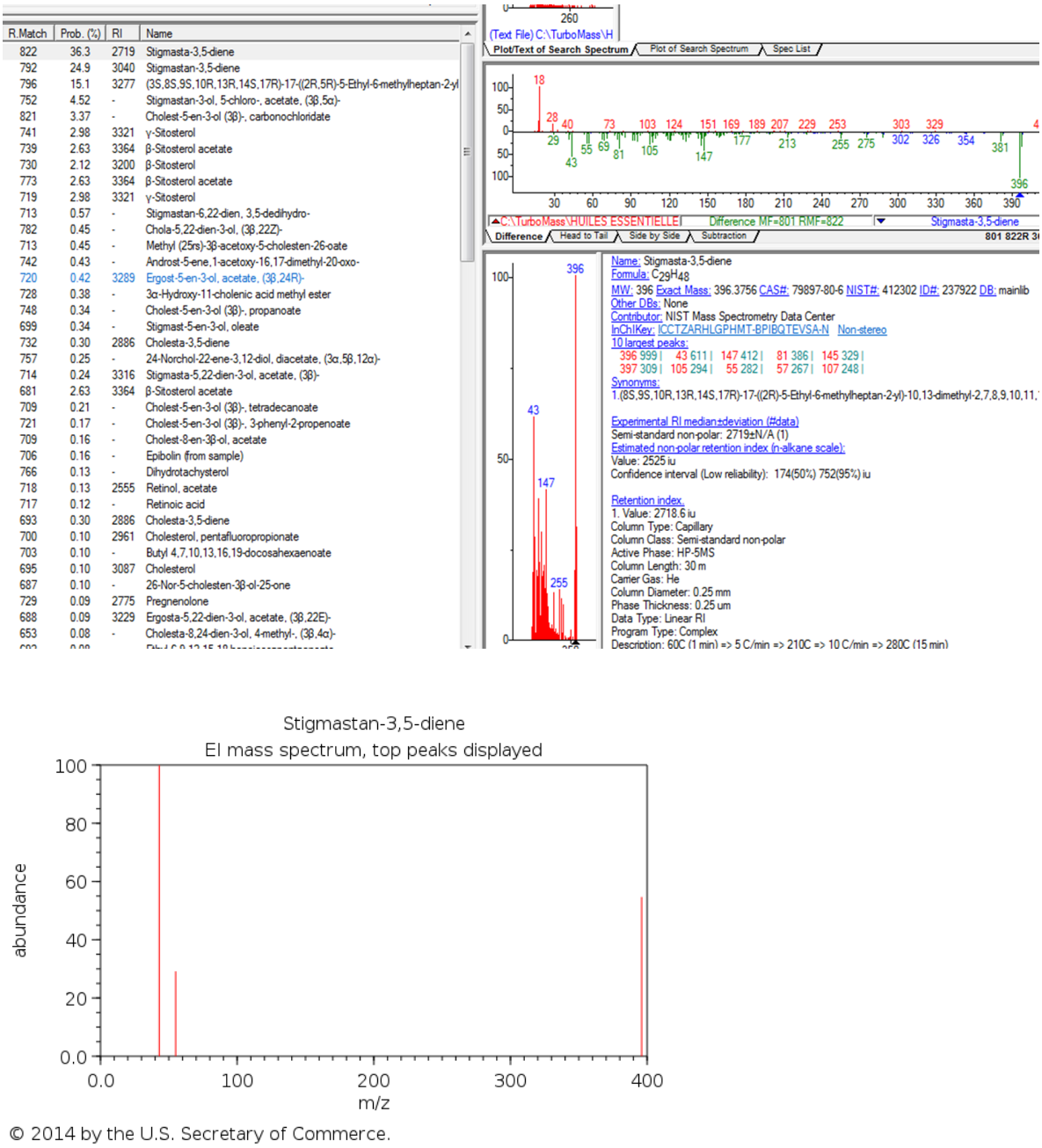


Figure 7. Mass Spectrometry GC-MS of Stigmasta-3,5-diene (above). The reference figure below is for the website of the National Center for Biotechnology Information website (National Center for Biotechnology Information (NCBI), 2024).

The peaks of the mass spectra for Squalene, as revealed by GC-MS on the left of Figure 9, are similar to the mass spectra peaks reported by the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024). The highest peak

is revealed at 69 Daltons, which is reported in our library on the left of Figure 9 and also on the right of Figure 9.

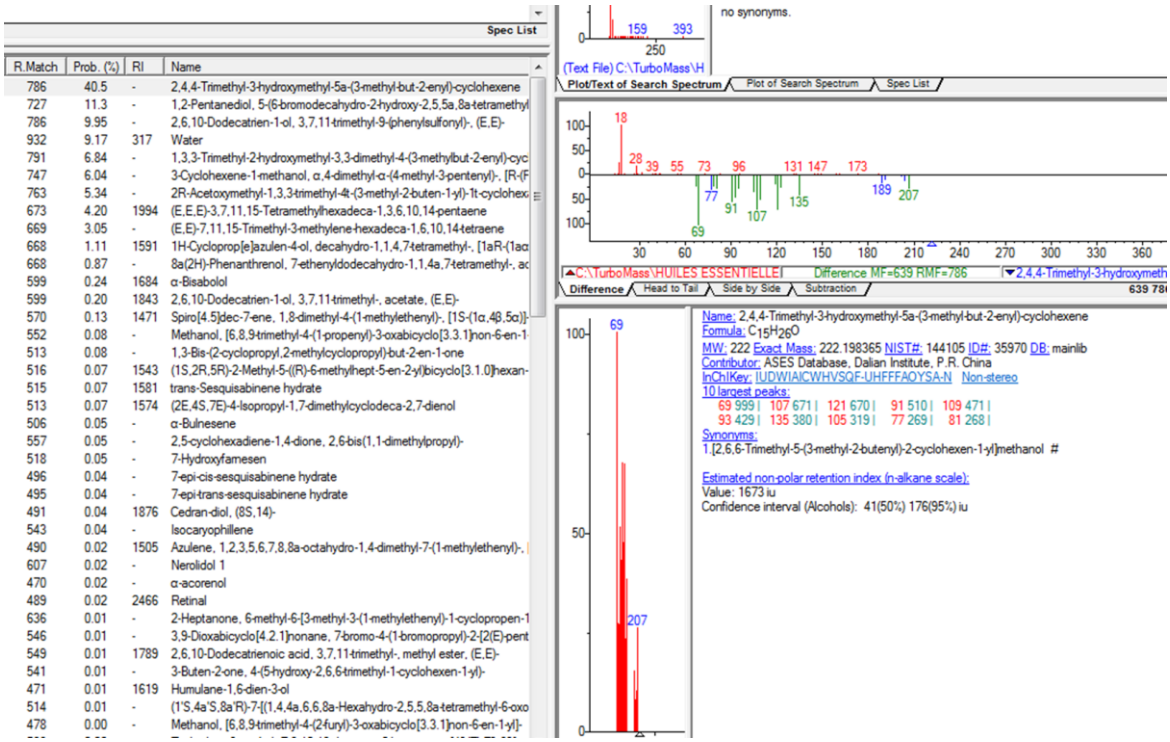


Figure 8. Mass spectrometry GC-MS of 2,4,4-Trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene.

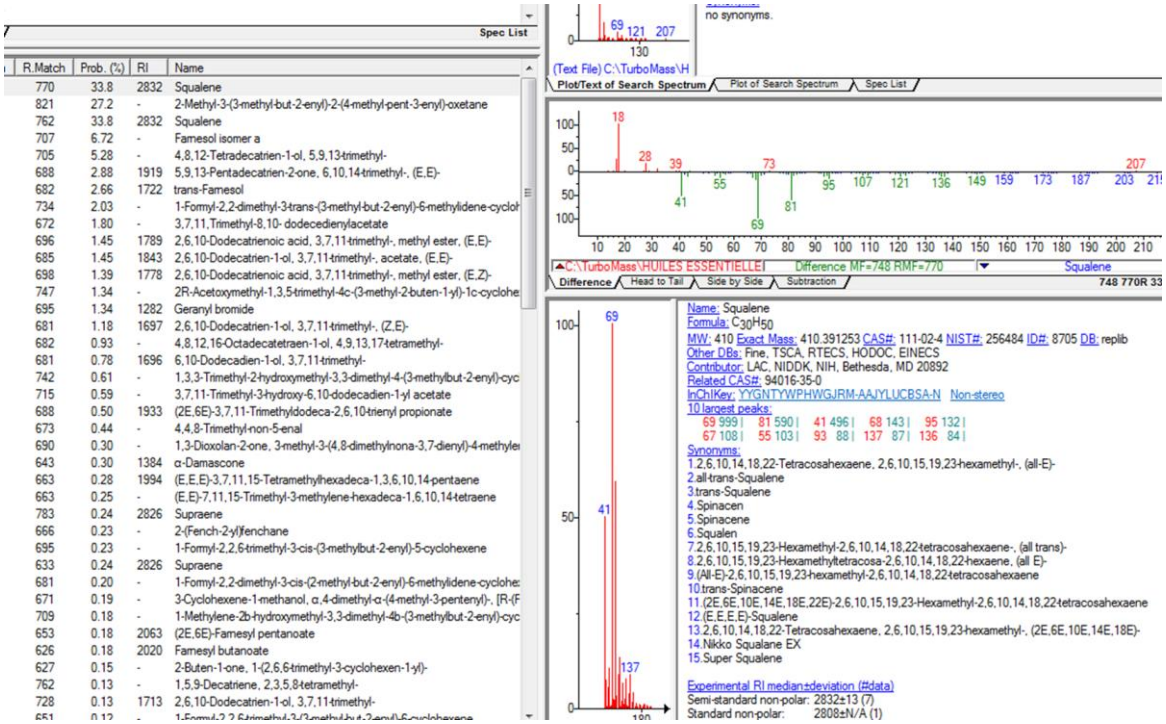


Figure 9. Mass spectrometry GC-MS of Squalene (above). The reference figure below is for the website of the National Center for Biotechnology Information website (National Center for Biotechnology Information (NCBI), 2024).

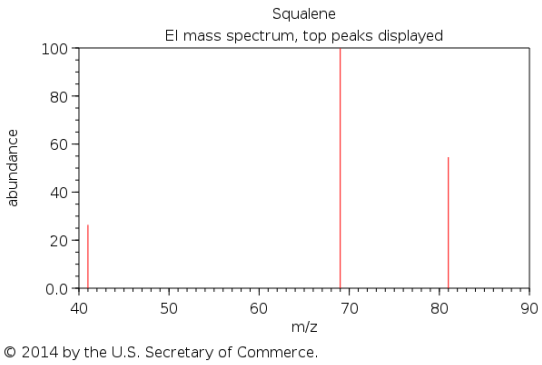
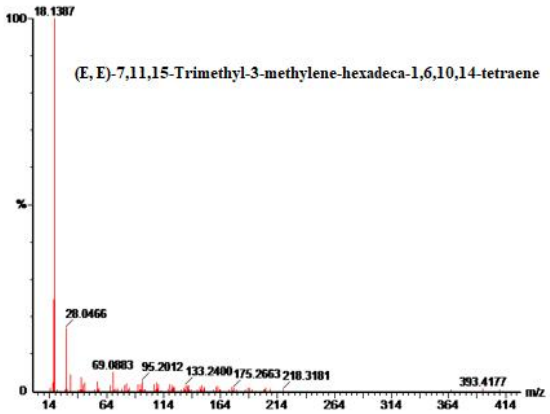


Figure 9. *Continue*.

The peaks of the (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene revealed by mass spectra GC-MS on the left of Figure 10 are similar to the peaks obtained by the National Center for Biotechnology Information shown in the right of Figure 10. The highest peak revealed at 69 Daltons is also reported by the library of the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024). The next peak at 95 Daltons is also similar to the peak reported by the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024) (Figure 10 right).

R.Match	Prob. (%)	RI	Name
744	13.9	-	(E,E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene
702	11.2	-	5,6-Azulenedimethanol, 1,2,3,3a,8,8a-hexahydro-2,2,8-trimethyl-, (3aα,8β,8aα)-
742	8.15	1843	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, acetate, (E,E)-
730	4.69	3031	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-(±)-
666	4.51	1876	Cedran-diol, (8S,14)-
728	4.34	1995	p-Camphorene
808	4.00	-	3-Cyclohexene-1-methanol, α,4-dimethyl-α-(4-methyl-3-pentenyl)-, [R-(R*,R*)]-
727	3.22	1960	m-Camphorene
637	2.85	-	1-Heptatriacotanol
674	2.74	1619	Humulane-1,6-dien-3-ol
664	2.63	-	α-Bulnesene
699	2.43	-	Isocaryophillene
631	2.33	-	2H-Pyran, 2-(7-heptadecynyloxy)tetrahydro-
647	2.15	-	α-acorenol
655	1.82	1505	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1α,7α,8aβ)]-
696	1.74	2302	2,6,10,14-Hexadecatetraen-1-ol, 3,7,11,15-tetramethyl-, acetate, (E,E,E)-
662	1.74	1492	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1α,7β,8aα)]-
630	1.61	-	Butyl 6,9,12,15-octadecatetraenoate
624	1.61	-	Methyl 5,11,14,17-eicosatetraenoate
711	1.74	1492	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1α,7β,8aα)]-
700	1.23	2234	(2E,6E,10E)-3,7,11,15-Tetramethylhexadeca-2,6,10,14-tetraen-1-yl formate
642	1.18	-	Pentacyclo[9.1.0.0(2,4).0(5,7).0(8,10)]dodecane, 3,3,6,6,9,9,12,12-octamethyl-, anti,anti,anti-
675	1.14	1493	1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1aα,7α,7aβ,7bα)]-
622	1.09	-	2,3,3-Trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone
636	1.01	-	7-Oxabicyclo[4.1.0]heptane, 2,2,6-trimethyl-1-(3-methyl-1,3-butadienyl)-5-methylene-
632	0.89	-	2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pentaene
624	0.75	-	4,6,6-Trimethyl-2-(3-methylbuta-1,3-dienyl)-3-oxatricyclo[5.1.0.0(2,4)]octane
622	0.72	-	2H-Cyclopropa[g]benzofuran, 4,5,5a,6,6a,6b-hexahydro-4,4,6b-trimethyl-2-(1-methylethenyl)-
617	0.67	-	Ethanone, 1-(1,2,3,4,7,7a-hexahydro-1,4,4,5-tetramethyl-1,3a-ethano-3aH-inden-6-yl)-
700	0.64	1441	1H-3a,7-Methanoazulene, octahydro-1,9,9-trimethyl-4-methylene-, (1α,3aα,7α,8aβ)-
683	0.64	1635	Hinesol
618	0.59	-	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester
614	0.54	-	Chola-5,22-dien-3-ol, (3β,22Z)-
627	0.50	-	Sandaracopimar-7,15-dien-6-one
628	0.48	1781	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)-
636	0.46	1486	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4aα,7α,8aβ)]-
614	0.46	-	Spiro[2.7]dec-4-ene, 1,1,5,6,6,9,9-heptamethyl-10-methylene-
687	0.44	1490	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-



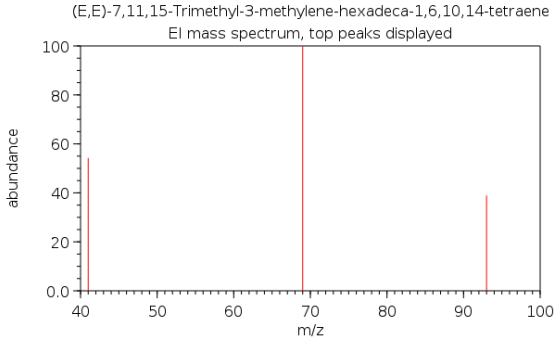


Figure 10. Mass spectrometry GC-MS of (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (above). The reference figure below is from the website of the National Center for Biotechnology Information (National Center for Biotechnology Information (NCBI), 2024).

In our current study, GC-MS analysis detected several compounds in the neem plant that are similar to those reported in other GC-MS studies. One such compound is stigmasta-3,5-diene (see Figure 7), which is a derivative of beta-sitosterol (Babatunde *et al.*, 2019). Beta-sitosterol is a promising anticancer agent for both chemoprevention and chemotherapy (Wang *et al.*, 2023). The compound 2,4,4-trimethyl-3-hydroxymethyl-5a-(3-methyl-but-2-enyl)-cyclohexene (Figure 8) detected in our study is suggested to be the related compound of 1,3,3-trimethyl-2-hydroxymethyl-3,3-dimethyl-4-(3-methyl but-2-enyl)-cyclohexene found in oil extracts of neem obtained from steam and solvent extraction methods with two solvents, ethanol, and hexane (Babatunde *et al.*, 2019).

Squalene (Figure 9) was also detected in a study by Babatunde *et al.* (2019), which focused on the chemical composition of steam and solvent crude oil extracts of neem. As well, Tulashie *et al.* (2021) conducted a study on the characterization and assessment of the toxicity of neem extracts obtained using two different methods against fall armyworm (FAW) larvae. The study revealed that squalene is the major compound present in the neem seed oil extract, constituting 21.61048%. This compound may be responsible for the pesticide activity observed in the neem oil extract (Tulashie *et al.*, 2021).

This study is the first to report the presence of compounds 13-Tetradecen-11-yn-1-ol and (E, E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene in neem oil obtained by cold pressing. The study conducted by Murniasih *et al.* (2023) found that the active compound Stigmasta-3,5-diene reported in antibacterial activity by Khan *et al.* (2016) was revealed in GC-MS analysis of ethyl acetate extracts of the marine invertebrate Lithistid sponge.

In a study conducted by Balasubramanian *et al.* (2014) the methanolic extract of neem leaves was obtained using a Soxhlet extractor and analysed through GC-MS. The analysis identified five major compounds in the extract: phytol, linolenic acid, homo-γ-linolenic acid, palmitic acid, and tridecylic acid. In this current study, *n*-hexane was selected as the fractional solvent for GC-MS analysis because it is inert and almost insoluble in water (~0.01 g/L). It provides excellent chromatography with split-less injections onto DB5 columns. With a boiling point of 69°C, it can take advantage of the 'solvent trapping effect' to produce good peak shapes with early/middle elutes when an initial column oven temperature of 45°C is used (Strutt, 2017). Also, hexane can produce good recoveries of low levels of semi-volatiles and often gives cleaner extracts. Peak shapes can be better than for dichloromethane with a split-less injector for some early/medium eluting compounds as you can use the 'solvent trapping effect' at a higher initial column oven temperature. Hexane is very inert and, it cannot react with analytes. However, dichloromethane for example can generate chlorine acid hydrogen (HCl) in a hot injector causing degradation and forming active sites in the injector and column (Strutt, 2017)

In this current study, regarding the diffusion disk method for antibacterial analysis, it has been observed that neem oil obtained through cold pressing is not effective in fighting bacteria due to the low concentration of active compounds, and also the GC-MS analysis showed that the oil has a high concentration of fatty alcohol and phytosterol, while antibacterial active elements such as terpenes, phenolics, saponins, tannins glycosides are present in lower quantities or are absent. Similar results were also found in the study of Cesa *et al.* (2019) when neem oil obtained by cold-pressure had weak or no activity against the bacterium *Helicobacter pylori*, and the fungi strains *Candida* spp. and *Malassezia furfur*.

The absence of antibacterial activity in our study could be also linked to the diffusion of the oil because it is hydrophobic. It also can be explained that the oil is mixed with other compounds which facilitate their diffusion, which shows a negative activity against such bacterial strains. It could also be due to the resistance of these strains. Moreover, if the concentration of crude oil used in the antibacterial test is increased, positive results could be obtained. However, it is possible to extract active ingredients with effective antibacterial properties through the fractionation of the oil based on the polarity of the solvent used to obtain an essential oil or organic extracts. The neem oil obtained from a company by cold pressing was tested for its

effectiveness against *Helicobacter pylori*. The oil was treated with diethyl ether and aqueous methanol, and then after fractioning, organic extracts were obtained. These organic extracts from the oil showed a significant ability to kill the bacteria (Blum *et al.*, 2019). The extract was tested against *H. pylori* strains, and its effectiveness was measured by its ability to inhibit (MIC) and kill bacteria (MBC). The extract's bactericidal activity was dependent on its concentration and incubation time. At 75-105 µg/mL, no bacteria were detected after 6 hours. (Blum *et al.*, 2019). Accordingly, many studies showed that neem essential oil has multiple biological properties including anti-inflammatory, antibacterial, antifungal, antipyretic, diuretic, hypoglycaemic, antiarthritic, anti-gastric ulcer, antimalarial, and spermicidal activities, and is more effective than vegetable oil (Ghosh *et al.*, 2015). In a study by Upadhyay *et al.* (2010), the antibacterial activity of neem essential oil was moderate against five Gram-positive bacteria *B. cereus*, *Lactobacillus acidophilus*, *Micrococcus luteus*, *S. aureus*, and *Streptococcus pneumoniae*; and two Gram-negative bacteria *E. coli* and *Klebsiella pneumoniae*. Neem essential oil effectively kills *L. acidophilus* and *S. pneumonia* bacteria, with a low MIC value of 0.125µL/mL against *L. acidophilus*. The minimum bactericidal concentration (MBC) value was also lowest against *L. acidophilus* and *S. pneumonia* at 0.25µL/mL. Neem oil's MIC value against *L. acidophilus* was lower than common antibiotics like tetracycline, ampicillin, and ciprofloxacin. Similarly, its MBC value against *L. acidophilus* was lower than these antibiotics. They conclude that neem essential oil could be an alternative to antibiotics for treating bacterial infections caused by *L. acidophilus* and *S. pneumonia* (Upadhyay *et al.*, 2010).

The products extracted from the seeds of neem are used in the control of pests and agricultural diseases, where they mainly act to inhibit the development of insects and mites. They contain insecticidal compounds, belonging to the group of limonoids known as meliacins or tetranortriterpenoids (azadirachtin), the principal representatives of the class of terpenoids that show insecticidal action (Schlesener *et al.*, 2013). Other findings about neem seed oil showed that it is the oil generally used with a mixture of some natural and synthetical chemical products to get good potential activity, such as insecticidal potency against pests inhibiting the development of insects and mites (Choupanian *et al.*, 2017), preserving fruit quality (da Silva *et al.*, 2020), prevention of cancer development and progression (Alzohairy, 2016), showing antipyretic and anti-inflammatory activities (Arora *et al.*, 2011; Kumar *et al.*, 2012; Naik *et al.*, 2014) and antibacterial activity against *Staphylococcus aureus* when the oil is mixed with various crosslinking agents (glyoxal/glycol, citric acid, 1,2,3,4-butane tetracarboxylic acid) (Joshi *et al.*, 2010). Hence, in a study by Choupanian *et al.* (2017), neem oil (3% azadirachtin), mixed with nanoemulsion formulations (non-ionic and ionic alkyl polyglucoside surfactant and, non-ionic and ionic polysorbate surfactant) and water significantly increased the mortality of two serious pest insect species *Sitophilus oryzae* and *Tribolium castaneum* when compared to non-formulated neem oil. They concluded that neem oil was found to be effective against *S. oryzae* and *T. castaneum* pests in stored products by using nanoemulsion formulations (Choupanian *et al.*, 2017). In another study, to maintain the quality of Guava fruit that is susceptible to attack by pests and diseases both pre-and post-harvest, the employment of techniques such as the use of neem oil-based products and chitosan (poly (1-4) 2 amino 2-deoxy p-D glucan) (biopolymers derived from polysaccharides) solution together with cold storage was used (da Silva *et al.*, 2020). Following the physical, chemical, and enzymatic analysis (such as the antioxidant analysis) of the Guava fruit during pre-harvest and storage, neem oil (0.5%) + chitosan (1%) proved to be effective in preserving and prolonging the quality of the guava fruit during storage for 8days at 24 ± 1 °C, and 16days at 10 ± 1 °C. They conclude that the application of neem oil (0.5%) + chitosan (1%) during pre-harvest in the guava fruit was efficient in preserving fruit quality at temperatures of 24 ± 1 °C and 10 ± 1 °C (da Silva *et al.*, 2020).

In the same way, Alzohairy, (2016) reported that neem and its compounds may prevent cancer by affecting various cell signalling pathways, though the exact mechanism is unclear. Polysaccharides G1A and G1B present in bark extracts have good antitumor activity (Gupta *et*

al., 2019). Neem has ingredients that help activate tumour suppressor genes and stop the activity of genes that cause cancer, like VEGF, NF-κB, and PI3K/Akt. Neem also helps activate apoptosis and slow down the NF-κB signal, and the cyclooxygenase pathway. Research shows that neem is a good way to help stop cancer and activate tumour suppressor genes (Alzohairy, 2016).

The neem seed oil contains limonoids, including nimbolide and azadirachtin (Sarkar *et al.*, 2021). Azadirachtin can trigger cell death through apoptosis and autophagy by activating the caspase cascade (Srivastava *et al.*, 2012). Especially, less p21 enhances the activation of the caspase pathway in the presence of azadirachtin (Srivastava *et al.*, 2012; Sarkar *et al.*, 2021). Neem extracts and seed oil can help protect cells by removing free radicals, reducing damage caused by ROS, normalizing lipid peroxidation, reducing cell death, and increasing the number of CD4+ and CD8+ T-cells (Sarkar *et al.*, 2021).

Earlier findings showed anti-inflammatory and antipyretic activities (Naik *et al.*, 2014) of neem seed oil, and chemo-preventive effects of leaf extract (Arora *et al.*, 2011). Neem seed oil was tested on albino rats to evaluate its pain-relieving effects. Results showed significant pain relief at 1 or 2 ml/kg doses, with a dose-dependent effect (Kumar *et al.*, 2012). The study carried out by (Naik *et al.*, 2014) showed neem seed oil reduces swelling in albino rats with hind paw oedema caused by carrageenan. Higher doses (up to 2mL/kg body weight) result in greater reduction with 53.14% inhibition in 4th hour after injection (Naik *et al.*, 2014). Results of the study of Ilango *et al.*, (2012) conclude that the treated animals with 100mg/kg dose of carbon tetrachloride extract (CTCE) of neem fruit skin and isolated ingredient azadiradione showed significant antinociceptive and anti-inflammatory activities respectively (Ilango *et al.*, 2012).

Natural products, due to their eco-friendliness and non-toxicity, hold great promise for niche applications such as medical and healthcare textiles. In the study of (Joshi *et al.*, 2010), neem seed extract was applied to the cotton fabric using different crosslinking agents, and the antibacterial activity of the finished fabrics was evaluated quantitatively against the Gram-positive bacteria *Staphylococcus aureus*. They used extracts of neem seeds (vegetable oil) when mixed with some chemicals such as cotton, glyoxal/glycol, citric acid, and 1,2,3,4-butane tetracarboxylic acid (BTCA) (crosslinking agents). The results showed significant antibacterial activity of seed extract-treated fabric with various crosslinking agents after one wash. In the experiment, the mixtures of cotton + glyoxal/glycol + seed extract (l0 %w/v), cotton + citric acid + seed extract (10% w/v), and cotton + BTCA+ seed extract (10% w/v) showed high antibacterial activity (more than 99%) against *Staphylococcus aureus* respectively with the values of 99.9, 99.0, 99.5 (%) compared to the control samples which showed less activity against the tested bacteria with values of 42.0, 13.0, 18.0 (%). They concluded that neem seed extract-treated cotton fabric exhibits effective and durable antibacterial activity even after multiple washes (Joshi *et al.*, 2010). Ethanolic extract of neem seed oil showed the highest free radical scavenging activity at 200µg/mL with 66.34 ± 0.06% (Nahak & Sahu, 2011).

5. CONCLUSION

GC-MS analysis of neem seed oil obtained by cold-pressure showed many effective compounds, among them two major and active substances first reported in the neem oil namely the phytosterol Stigmasta-3,5-diene (the derivate from B-Sitosterol) and the fatty alcohol Tetradecen-11-yn-1-ol. The antibacterial test results were negative against the food-borne pathogens *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Salmonella* sp., and the soil bacterium, *Bacillus subtilis*. This suggests that cold-press extraction may not be effective against these strains due to the high levels of fatty alcohol and phytosterols. Resistance of these strains might also be a reason. Moreover, increasing the concentration of crude oil used in the antibacterial test could yield positive results. However, the individual compounds identified through GC-MS have potential biological activities and therapeutic uses. The study recommends further research into different extraction methods, such as steam distillation, to extract essential oil and explore its potential

medicinal applications, including its antibacterial, anti-insecticidal, anti-cancer, antimalarial, and antipathogen properties. When fractionating neem oil, it's important to consider both the extraction method, which aims to obtain essential oil, and the polarity of the extraction solvent in order to isolate components with effective biological activities. This study's results help us understand the impact of the extraction method of neem seed oil on biological activity. Additionally, the study reports that various bioactive components present in the neem plant could be used as herbal alternatives for treating various illnesses.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

BMO: Investigation, Resources, Software, Formal Analysis, and Writing - original draft. **BMO** and **OB:** Materials, Method, Analysis, Interpretation. **OB** and **IB:** Visualisation, Critical Review. **SY** and **BBO**: Supervision, Visualization, Validation.

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Research Article

Physiologically active compounds in the fruits of *Smilax excelsa* L.

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Abstract: Currently, in all areas, especially in the fields of food and medicine, eco-friendly consumption materials are highly preferred. Therefore, the study aims to determine the physiologically active compounds and radical scavenging activity (RSA) of the bioextracts prepared with 70% ethanol (BE-I) and distilled water (BE-II) from the ripe fruits of *Smilax exselca* L. (Smilacaceae) plant which grows in the northwestern region of Azerbaijan by physicochemical methods ((GC/MS), ultraviolet (UV) spectrophotometers). As a result of the research, 35 compounds were observed in BE-I and 30 compounds in BE-II, the amount of main physiologically active compounds was 37.14% with 12 compounds in BE-I, and 19.86% with 6 compounds in BE-II. For instance, antioxidant properties of 5-hydroxymethylfurfural ($C_6H_6O_3$), 6-Octadecenoic acid ($C_{18}H_{34}O_2$), hepta-2,4-dienoic acid, methyl ester ($C_8H_{12}O_2$), antibacterial traits of ascaridole epoxide ($C_{10}H_{16}O_3$), α-D-Galactopyranose,6-O-(trimethylsilyl)-cyclic 1,2:3,4-bis methylboronate ($C_{11}H_{22}B_2O_6Si$), hexadecanoic acid, methylester ($C_{17}H_{34}O_2$), flavoring compounds of 2H-Pyran, 3,6 Dihidro-4–methyl-2-2-Methyl-propenyl ($C_{10}H_{16}O$), E-10-Dodecen-1-ol propionate ($C_{15}H_{28}O_2$), anticancer properties of 2-Trifluoroacetoxydodecane ($C_{14}H_{25}F_3O_2$), anti-inflammatory properties of silicicacid, diethyl bis(trimethylsilyl) ester ($C_{10}H_{28}O_4Si_3$), antifungal traits of D-Mannitol,1-decylsulfonyl ($C_{16}H_{34}O_7S$) were determined. The DPPH method was used to determine the free radical scavenging activity (RSA), which came out to be $RSA_{(BE-I)}$=62.5±0.8% and $RSA_{(BE-II)}$=54.7±1.3%.

1. INTRODUCTION

Smilax excelsa L. (Smilacaceae), one of the economically and medicinally important plants, is found in the northwest of Azerbaijan. Physiologically active chemicals and pharmacological qualities of *Smilax excelsa* L. fruits grown in Azerbaijan have not been studied. One species of *Smilax excelsa*, a liana-type plant, primarily grows in the Caucasus and Azerbaijan. The leaves, fruits, and roots of this plant have been used medicinally for centuries by various cultures. Additionally, shamans traditionally used it to treat dermatitis, psoriasis, and other skin conditions (Əsgərov, 2005) . People in Central and South America utilized the root of this plant as a diuretic and tonic booster (Əzizov *et al*., 2020; Elina, 1993).

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Currently, this plant's leaves, roots, and fruits are frequently used in scientific medicine to treat and avert diseases of the gastrointestinal tract(Cox *et al*., 2005), genitourinary (Pansumrit *et al*., 2022), nervous system disorders, and depression (Sayyaed *et al*., 2023) in the body. The presence of biologically active steroid saponins in the formulation of the remedies made from different plant parts accounts for their therapeutic action. Extracts of Smilax excelsa L. can be used to treat conditions affecting the kidneys, bladder, urinary system, pancreas, liver, and biliary tract (Bruno *et al*., 1985; Muravyova, 1981).

Many species of *Smilax excelsa* found in different parts of the world have been studied for their chemical composition. It was found that these species are abundant in physiologically active chemicals with notable antioxidant properties (Kretovich, 1989). Antioxidant capacity and components of Smilax species gathered from various locations by Turkish researchers were determined, in addition to certain phenological and morphological aspects. They concluded that smilax species' leaves and berries can be regarded as a significant source of naturally occurring antioxidants (Yildiz *et al*., 2018). Later the anthocyanin content of its fruit was studied, and pelargonidin 3-0-rutinoride (20.4 mg^{-1}) and cyanide 3-0-rutinoride (1.67 mg^{-1}) were discovered to be the major anthocyanins (Longo & Vasapollo, 2006). *Smilax excelsa* leaves, which have spread widely in the Black Sea region of Turkey, were found to have antioxidant properties. The antioxidant potential of the extracts was also assessed, and measures were taken for lipid peroxidation inhibition, metal ion chelation, DPPH, reduction of superoxide, hydroxyl radicals, and hydrogen peroxide, as well as radical scavenging competencies (Ozsoy *et al*., 2008). By comparing the antioxidant behaviors of water, infusion, ethanol, and ethyl acetate extracts to their total amount of polyphenol components, the extraction method's efficiency was calculated (Chen *et al*., 2000). Traditional Chinese medicine frequently uses the root and leaf of the *Smilax China plant*, an indigenous species of the *Smilax* genus, to treat inflammatory pain, rheumatic, diuretics, joint pain, and gout (Khan *et al*., 2009). In subsequent studies, the content of phenolic compounds in the root, stem, and leaf of Smilax China was 19.31%, 8.59%, and 44.27% respectively, in which 3-hydroxy benzoic acid predominated as the major phenolic compound and DPPH activity of the extract was determined to be 8.66 mg/ml, and the total phenolic content was 2,383.55 µg gallic acid equivalent/mL (Shim, 2012). Previous research determined the presence of 25 mineral elements in the vegetative and generative organs of the Smilax-excelsa L. plant, the amount of microelements Zn, Mn, Cr, Si was 0.679% in the leaf, 0.935% in the stem, 2.10% in the flower and 0.578% in the fruit. The fruits of the *Smilax* genus have been found to contain a high concentration of physiologically active compounds based on their biochemical composition, including carotene (0.69 mg%), carotenoids (7.35 mg%), ascorbic acid (103 mg%), α-tocopherol (5.62 mg%), and flavonoids (0.90%) (Xəlilov & Xəlilov, 2021)

Other researchers prefer mainly the root and stem parts of the plant. This causes considerable damage to the reduction of plant biodiversity and the ecosystem chain. The most commonly used part of this plant in folk medicine and household are the dry fruits of this plant. Therefore, we prefer to conduct research with fruit instead of working with other vegetative organs.

In light of the above, this significant plant native to Azerbaijan should be thoroughly studied to identify its physiologically active compounds and conduct research that could facilitate its application in scientific medicine.

2. MATERIAL and METHODS

2.1. Extract Preparation

For the experiment, ripe fruits of *Smilax excelsa* which belongs to the northern-west region of Azerbaijan were collected. The fruits were first washed with water, separated from seeds, dried, and then crushed. Then the crushed materials were subjected to extraction using distilled water (BE-II) and 70% ethanol (BE-I). A 500-milliliter flask was filled with a 50 gram of ground sample. Subsequently, the sample was mixed with 300 milliliters of distilled water. The mix was incubated for 30 minutes at a temperature of between 75 and 80°C in a water bath, after which the solution was filtered. Then, the remaining pulverized sample was mixed with 100

mL of distilled water and extracted for 15 minutes. After extraction, it was filtered and combined with the original extract. The residue in the flask was refilled with 100 mL of distilled water and extracted for an additional 15 minutes. It was extracted, filtered, and then mixed with the first extract. The extraction process in alcohol also was carried out in the same way. A device known as an SPT-200 Vacuum-Drier was used to powder both extracts (Azizov *et al*., 2021).

Taking into account the preparation of plant extracts at home, it is appropriate to prepare bioextracts with water and ethanol, because they are available to the population and their preparation is simple. Although other organic solvents are more effective, the use of this method for the population is inappropriate and does not guarantee safety.

2.2. Identification of Organic Compounds

Biologically active compounds in the extracts were identified by using an Agilent Technologies 6890 N Network CG System, a gas chromatograph equipped with a 5975 inert Mass Selective Detector spectrometer (USA). It also has an injection-split detector that was split/splitless, split - 100, low mass – 40, high mass – 400, and threshold 150. A 30-meter quartz capillary column named "HP-5MS 5% Methyl Siloxane" with an internal diameter of 0.25 mm and a stationary phase thickness of 0.25µm was used for the experiments. The analyses were carried out in temperature programming mode at a rate of 15ºC per minute between 50ºC and 280ºC. The temperature regime in the column was as follows: the beginning temperature of 50ºC that was constant for two minutes; a two-minute temperature rise from 15ºC to 200ºC; a ten-minute temperature rise from 15ºC to 280ºC; and a vacuum HiVac of 3.38e-005. Dilution was carrying by using a 1:1:2 methanol, chloroform to water combination. The velocity of He gas was1 milliliter per minute. Materials were identified using the standard mass spectroscopic NIST library. In total 33 minutes were spent on the analysis *(*Azizov *et al.,* 2022).

2.3. Radical Scavenging Activity (RSA) assay

The free radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Sigma-Aldrich, USA). A solution of 70 µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) was produced in methanol, and the absorption was then measured in a 3 ml cuvette using a UV-2700(Shimadzu, Japan), and the value of absorption was roughly 0.8211 at 516 nm. After extracting 0.5 ml of DPPH solution from that cuvette and adding 0.5 ml of 200 mgL^{-1} bioextract, the kinetics was observed after 20 minutes. The following formula was used to determine the DPPH radical scavenging activity: RSA% is equal to $(A_0\text{-}A_s)/A_0$ (Atayeva & Aslanov, 2022).

2.4. Statistical Analysis

The mean values of triplicates of the same sample comprise the results, and analysis of variance (ANOVA) was used for statistical analysis and differences between them consisted of $p < 0.05$

3. FINDINGS

Following the experiment, GC/MS was used to record 35 chemicals in BE-I and 30 compounds in BE-II. During the experiment, GC/MS recorded 35 chemical compounds in BE-I and 30 chemical compounds in BE-II. Each compound was identified individually and their medicinal characteristics were defined and chosen based on the literature that was accessible. In BE-1 and BE-2, the physiologically active chemicals that were selected comprised 37.14% and 19.86%, respectively. Physiologically active substances in the BE-I extract are listed in Table 1, and physiologically active compounds in the BE-II extract are given in Table 2, as no information was discovered about additional substances in the extracts. The mass spectrum results of the identified physiologically active compounds are shown in Figure 1.

Table 1. Physiologically active chemicals in contents of BE-I.

Peaks	RT	Compound name	Formula of compound	m/z	Peak area %	Therapeutic activity
1	6.940	2H-Pyran,3,6 Dihydro-4–methyl-2-(2-Methyl-propenyl)	$C_{10}H_{16}O$	98.07	5.42	Aromatic, flavoring (Karabagias *et al*., 2021)
2	7.240	2-Trifluoroacetoxydodecane	$C_{14}H_{25}F_3O_2$	282.18	0.47	Anticancer (Fadeyi *et al*., 2015)
3	10.369	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.03	20.19	Anticanser(Kelleci & Gölebatmaz, 2023; Antioxidant (Zhao *et al*., 2013)
4	10.575	6-Acetyl-β-d-mannose	$C_8H_{14}O_7$	222.07	1.02	Antimicrobial (Ezekwe & Chikezie, 2017
5	11.004	4-Hydroxylamino-6-methylpyrimidin-2(1H)-one	$C_5H_7N_3O_2$	141.05	41.19	Anticancer, anti-oxidant, anti-tumorigenic, anti-mutagenic, cytotoxic activity (Mohan *et al*., 2018)
6	11.257	Ascaridole epoxide	$C_{10}H_{16}O_3$	184.11	2.56	Antioxidant, antimicrobial, anticanser (El-Amier *et al*., 2023)
7	13.716	α-D-Galactopyranose,6-O-(trimethylsilyl)-cyclic 1,2:3,4-bis(methylboronate)	$C_{11}H_{22}B_2O_6S$	300.14	0.17	Antimicrobial (Shelly *et al*., 2015)
8	18.021	Carbazic acid, 3-pentylidene-, methyl ester	$C_7H_{14}N_2O_2$	158.11	1.26	Hypertension, vasodilator (Liu *et al*., 2009)
9	19.892	E-10-Dodecen-1-ol propionate	$C_{15}H_{28}O_2$	240.21	0.12	Aromatic, flavoring Mishra *et al*., 2021
10	20.686	Hexadecanoic acid, metyl ester	$C_{17}H_{34}O_2$	270.26	1.05	Antimicrobial (Ezekwe & Chikezie, 2017), Antifungal(Francis *et al*., 2021), Antibacterial (Shaaban *et al*., 2021), Anti-Inflammatory,(Othman *et al*., 2015) Antioxidant (Muflihunna *et al*., 2019)
11	26.791	D-Mannitol,1-decylsulfonyl	$C_{16}H_{34}O_7S$	370.20	1.68	Antimicrobial (Khromykh *et al*., 2022), Antifungal (Thankaraj *et al*., 2020), Antibacterial (Khromykh *et al*., 2022), Anti-inflammatory (Khromykh *et al*., 2022), Antioxidant (Khromykh *et al*., 2022)
12	29.497	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	296.13	0.3	Antibacterial, (Momin & Thomas, 2020)

Table 2. Physiologically active chemicals in contents of BE-II.

Peaks	RT	Compound name	Formula of compound	m/z	Peak area%	Therapeutic behavior
1	8.981	Levoglucosenone	$C_6H_6O_3$	126.03	2.35	Anticancer, antitumor (Delbart *et al*., 2022)
2	9.457	Hepta-2,4-dienoic acid, methyl ester	$C_8H_{12}O_2$	140.08	25.36	Antioxidant, antiproliferative (Fagbemi *et al*., 2022)
3	10.445	5-hydroxymethylfurfural	$C_6H_6O_3$	126.03	32.62	Anticanser (Kelleci & Gölebatmaz, 2023), Antioxidant (Zhao *et al*., 2013)
4	11.939	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268.24	1.26	Antimicrobial, antifungal, antioxidant (Fagbemi *et al*., 2022)
5	20.803	Hexadecanoic acid, methylester	$C_{17}H_{34}O_2$	270.26	3.41	Antimicrobial, (Ezekwe & Chikezie, 2017), Antifungal (Francis *et al*., 2021), Antibacterial (Shaaban *et al*., 2021), Anti-inflammatory, (Othman *et al*., 2015), Antioxidant (Muflihunna *et al*., 2019)
6	27.168	6-Octadecenoic acid	$C_{18}H_{34}O_2$	282.25	14.62	Antioxidant (Alaribe *et al*., 2020)

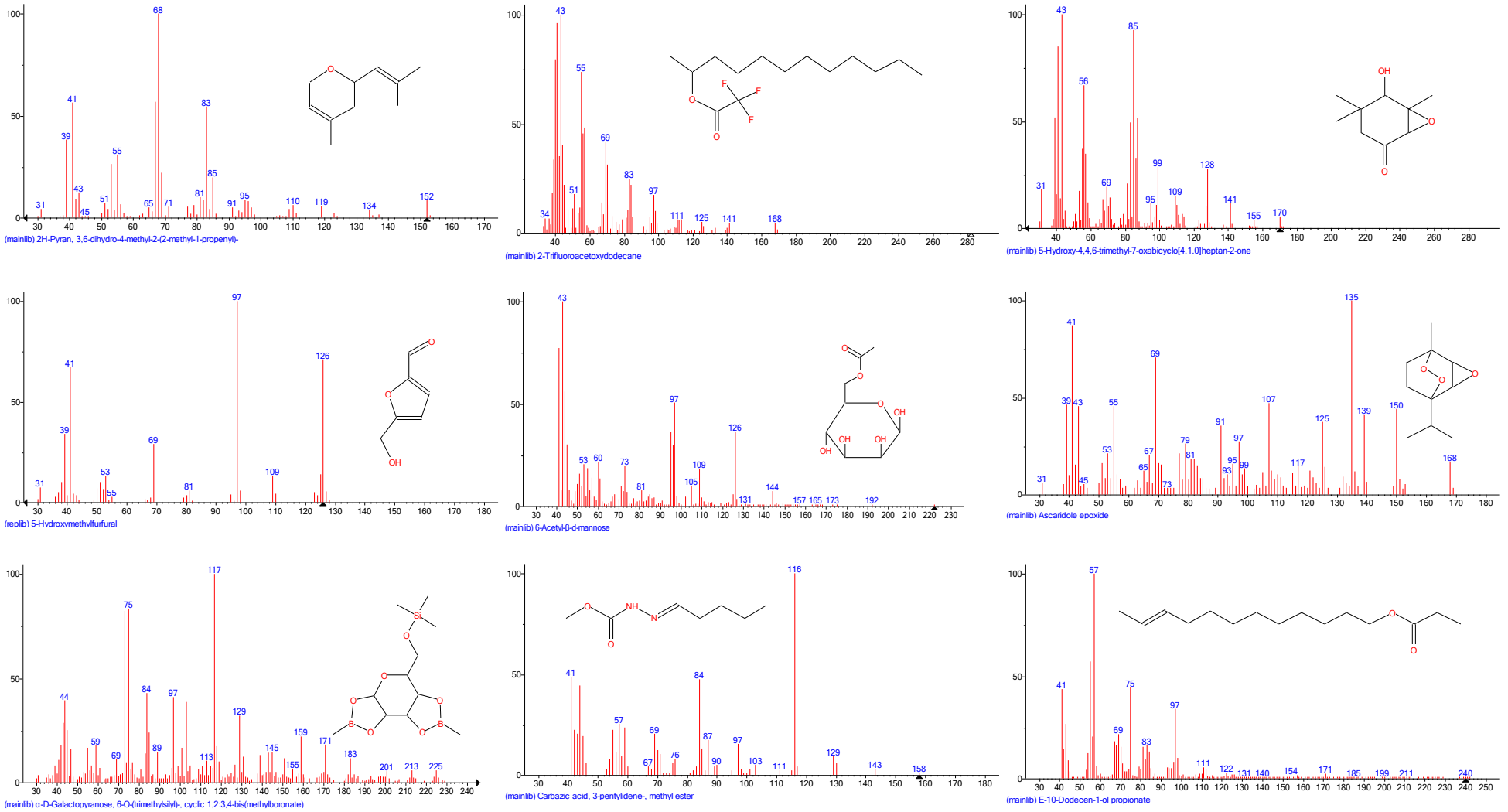
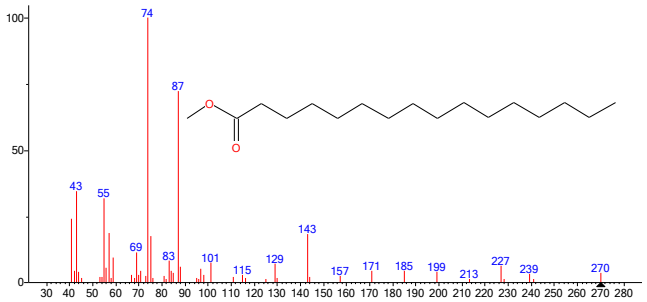
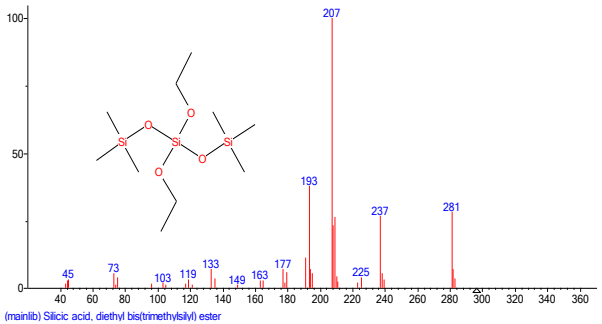
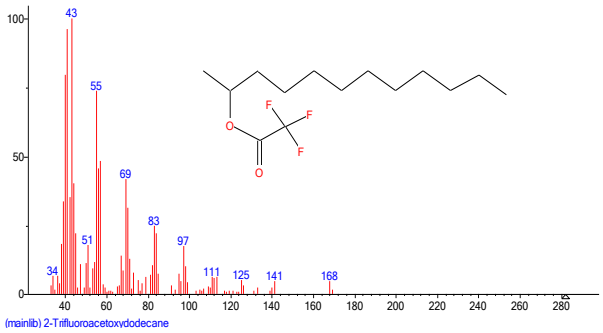
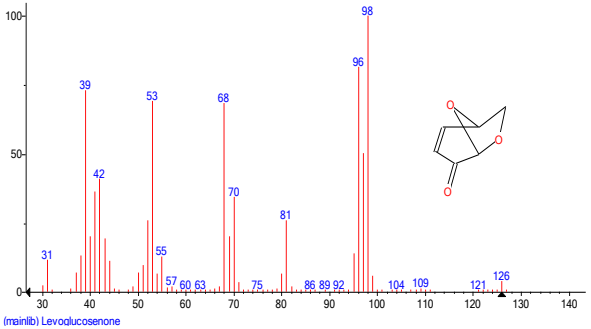


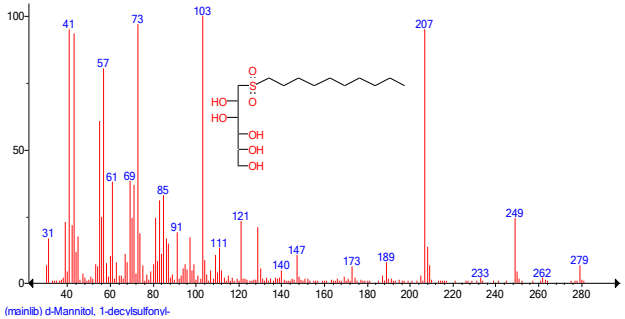
Figure 1. Mass spectra of physiologically active compounds shown in Table 1 and Table 2.

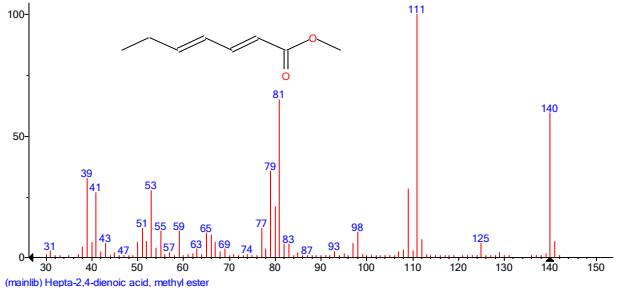












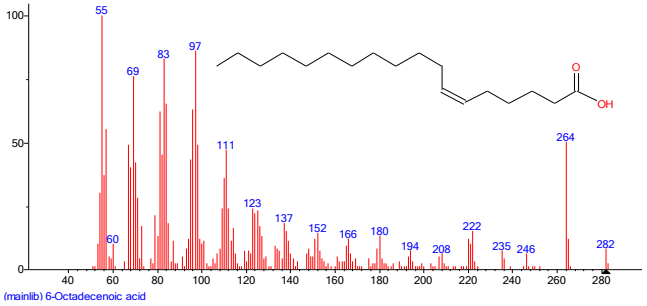


Figure 1. Continues.

The outcomes of the investigation revealed that the bioextract made with 70% ethanol (BE-I) consisted of 12 physiologically active compounds, however, the bioextract made with distilled water (BE-II) contained 6 physiologically active compounds. Physiologically active compounds, which in their separate states have substantial medical value in the body, were present in both bioextracts. These molecules exhibit distinct physiological implications. The most often utilized chemical in the production of nutmeg syrop is 2H-Pyran,3,6 Dihydro-4-methyl-2-(2-Methyl-propenyl), also called nerol oxide. This chemical, which is mostly present in flowers and gives them a sweet, pleasant scent, is produced when the monoterpene nerols oxidize It was previously employed in the manufacture of wine (Karabagias *et al*., 2021). Furthermore, it was discovered that flavoring ingredient E-10-Dodecen-1-ol propionate is a considerable component (Mishra *et al*., 2021). With a high peak area in both extracts, 5-hydroxymethylfurfural exhibits anticancer and antioxidant qualities (Kelleci & Gölebatmaz, 2023; Zhao *et al*., 2013). According to earlier research, *Smilax excelsa* fruits contain considerable levels of 6-Acetyl-β-d-mannose, which has been shown to have antibacterial properties (Ezekwe & Chikezie, 2017). Indian scientists conducted research that led to the discovery of 4-Hydroxylamino-6-methylpyrimidin-2(1H)-one, a chemical with anticancer, antioxidant, anti-tumorigenic, anti-mutagenic, and cytotoxic characteristics (Mohan *et al*., 2018). Research by Nouf S. Zaghloul and others has demonstrated the antioxidant, antibacterial, and anticancer effects of Ascaridole epoxide, the predominant component of the *Smilax excelsa* fruit extract (El-Amier *et al*., 2023). In comparison to amoxicillin, the antimicrobial activity of α-D-Galactopyranose,6-O-(trimethylsilyl)-cyclic 1,2:3,4-bis(methylboronate) encountered in BE-1 against gram positive bacteria demonstrates that it possesses beneficial antibacterial properties and could potentially be used for medicinal purposes in the future (Shelly *et al*., 2015.). The following substances have antioxidant, antimicrobial, antifungal, anti-inflammatory and antibacterial properties: hexadecanoic acid, methyl ester, D-mannitol,1-decylsulfonyl (Francis *et al*., 2021; Khromykh *et al*., 2022; Muflihunna *et al*., 2019; Othman *et al*., 2015; Shaaban *et al*., 2021; Thankaraj *et al*., 2020) Vasodilators and hypertension are two illnesses for which carbazic acid,3-pentylidene, methyl ester is utilized (Ezekwe & Chikezie, 2017; Parveen *et al*., 2013). Levoglucosenone , which was identified in BE-2, and 2-Trifluoroacetoxy dodecane, which was found in BE-1, both have anticancer and antitumor characteristics (Delbart *et al*., 2022; Fadeyi *et al*., 2015).While examining the *Dillenia scabrella* species leaves and bark, Monim *et al*. found Silicic acid diethyl bis(trimethylsilyl) ester, and highlighted the plant's antibacterial properties (Momin & Thomas, 2020.). Because of its antifungal properties, 7-Methyl-Z-tetradecen-1-ol acetate can lower fungus biomass by 36–54%. High levels of antibacterial, antioxidant, and antiproliferative activity were demonstrated by Hepta-2,4-dienoic acid, methyl ester, 6-Octadecenoic acid, and 7-Methyl-Z-tetradencen-1-ol-acetate that were discovered in BE-II (Alaribe et al., 2020; Fagbemi *et al*., 2022; Sana *et al*., 2016). 6-Octadecenoic acid molecule exhibits powerful antioxidant capabilities, including the ability to scavenge 2,2-diphenyl-l-picrylhydrazyl (DPPH) radicals, inhibit the peroxidation of lipids, and work in concert with iron-reducing activity (Dong *et al*., 2019).

Studies have discovered that the fruit of Smilax excelsa has a broad spectrum of physiologically active compounds and may be utilized to treat and avoid several illnesses. In the subsequent experiment, the fruit of the *Smilax excelsa* plant had been evaluated for its radical scavenging activity (RSA) using an extract made over the entire physiological ripening process. Figure 2 depicts the RSA study's kinetics, which was analyzed.

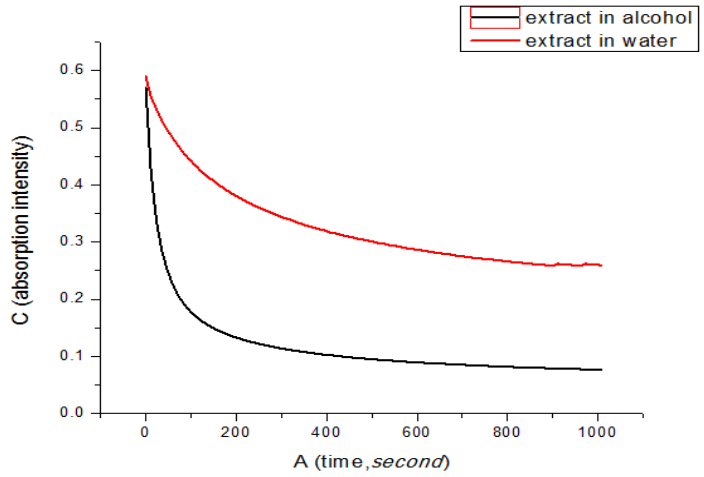


Figure 2. Kinetics of radical scavenging activity of the extracts

As seen from the kinetics of RSA value of BE-I was higher than BE-II which were $RSA_{(BE-I)}$=62.5±0.8% and $RSA_{(BE-II)}$=54.7±1.3%. The free radical scavenging activity of BE-I was also considered because the amount of bioactive substances identified in BE-I was greater than the amounts of substances determined in BE-II. As was known from Figure 2, in the kinetics of BE-I extract at the 100th second, the absorption curve from 0.6482 to 0.2148 decreased dramatically. In contrast, the kinetic curve of the BE-II extract changed gradually and slowly from 0.6482 to 0.3568 in intensity until the 300th second. The radical scavenging activity of both extracts was greater than *Citrus sinensis* RSA=65.74 ± 1.98, *Cocus nucifera* RSA=153.8 %, *Citrus anrantifolia* RSA=55.58 ± 1.00 in the study of Abdur Rauf *et al*., equivalent to the amount of dry powder in 3 ml cuvette. But *Citrus limonum* RSA=38.51 ± 1.98, *Mangifera indica* fruit RSA=44.7%, *Tomato* RSA=44.65, *Watermelon* RSA=40.90 values got smaller values than the radical scavenging activity of both extracts. The radical scavenging activity of *Malus Sylvestris (L.)* was high compared to RSA=56.7±10.2 % BE-I compared to less BE-II. and BE-I extracts (Mustafa *et al*., 2018; Prakash *et al*., 2011; Rauf *et al*., 2014; Shahzad *et al*., 2014).

4. DISCUSSION and CONCLUSION

BE-I and BE-II, which were derived from the fruit of the *Smilax excelsa L.,* have been reported to contain 14 physiologically active chemicals with antibacterial, antifungal, carcinogenic, and antioxidant properties. As can be seen from Table 1 and Table 2, the number of medicinally important substances in the aqueous extract is double less than the number of substances in the alcoholic solution. That is why the antioxidant behavior of these bioextracts varies, with BE-I having a higher activity than BE-II. So that having $RSA_{(BE-I)}$=62.5±0.8% and $RSA_{(BE-II)}$=54.7±1.3% provides us with the suitability of these bioextracts for therapeutic usage at home condition. The bioextracts produced from the fruit can be employed in the confectionery sector and in the production of alcoholic and non-alcoholic beverages due to the presence of two aromatizing and flavouring compounds. Both extracts have strong antioxidant properties, making them useful for both disease prevention and treatment.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Vafa Atayeva: Methodology Investigation, Finding Materials, Extraction and Writing and antioxidant activity of extractions. **Farhad Azizov**: Interpretation of Results, Writing -original

draft. **Zarbali Khalilov**: Extraction, Activity Experiments. **Nurmammad Mustafayev**: Supervision and Validation**. Hilal Imali**: GC/MS Experiments.

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Research Article

Chemical composition, antioxidant properties, and enzyme inhibitory activities of methanol extract from *Sideritis montana* subsp. *montana* using ultrasound-assisted extraction

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Abstract: Natural products are valuable sources of bioactive compounds with therapeutic potential. This study investigated the chemical composition, antioxidant properties, and enzyme inhibitory activities of the methanol extract from *Sideritis montana* L. subsp. *montana* obtained via ultrasound-assisted extraction. The extraction yielded 5.37%, with a total phenolic content of 63.27 mg GAEs/g extract and a total flavonoid content of 58.32 mg REs/g extract. Chlorogenic acid (563 µg/g extract), luteolin 7-glucoside (513 µg/g extract), and hyperoside (511 µg/g extract) were the most abundant phenolics. Moderate levels of luteolin and hydroxybenzoic acids were also identified. Antioxidant activity was most pronounced in the phosphomolybdenum assay (428.52 mg TEs/g extract), followed by the CUPRAC (217.40 mg TEs/g extract) and FRAP (171.33 mg TEs/g extract) assays, demonstrating strong reducing power. Radical scavenging assays (DPPH: 122.76 mg TEs/g, ABTS: 140.41 mg TEs/g) showed moderate efficacy, while ferrous ion chelation was weak (6.62 mg EDTAEs/g extract). Enzyme inhibition assays indicated potent α-glucosidase (753.81 mg ACEs/g extract) and α-amylase (274.95 mg ACEs/g extract) inhibition, suggesting antidiabetic potential. Tyrosinase inhibition (68.56 mg KAEs/g extract) points to possible dermatological applications, though acetylcholinesterase (2.08 mg GALAEs/g extract) and butyrylcholinesterase (0.45 mg GALAEs/g extract) inhibition was minimal. The results emphasize the bioactive potential of *S. montana* subsp. *montana*. Future studies should explore its bioactivity in vivo and identify synergistic effects among its phenolic compounds to further validate its therapeutic applications.

1. INTRODUCTION

In recent years, medicinal plants have garnered significant interest due to their bioactive compounds with antioxidant potential (Grzegorczyk *et al*., 2007; Miliauskas *et al*., 2004; Mohamed *et al*., 2010). Antioxidants are known to play a crucial role in safeguarding cells from

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oxidative stress, a condition that can lead to cellular damage (Gião *et al*., 2007). Medicinal plants are rich sources of various natural substances, including phenolic acids, flavonoids, terpenoids, vitamins, and tannins, all of which contribute to their antioxidant properties (Ai-Li & Chang-Hai, 2006; Bouayed *et al*., 2007). Notably, plants belonging to the Lamiaceae family have been widely investigated for their strong antioxidant and free radical scavenging activities (Barros *et al*., 2010; Erdemoglu *et al*., 2006).

Tyrosinase is an enzyme containing copper that catalyzes the *o*-hydroxylation of monophenols and subsequently oxidizes o-diphenols to form o-quinones (Zhou *et al*., 2017). While this process is crucial for melanin production in mammals and browning in plants and microbes, excessive melanin synthesis in human skin can lead to hyperpigmentation. Therefore, inhibiting tyrosinase activity represents a significant therapeutic approach for managing skin discoloration (Eruygur & Uçar, 2018). In a similar vein, Alzheimer's disease (AD), a chronic neurodegenerative disorder, is linked to reduced acetylcholine levels, a vital neurotransmitter in the brain. The enzymes acetylcholinesterase and butyrylcholinesterase break acetylcholine into choline and acetic acid, and their inhibition is a core strategy in AD treatment (Eruygur & Uçar, 2018; Ertaş *et al*., 2014). However, the side effects associated with synthetic inhibitors have driven the search for alternative, natural cholinesterase inhibitors. Diabetes mellitus, another complex metabolic disorder, frequently results in severe complications. Postprandial hyperglycemia, caused by glucose absorption in the digestive tract, is a major challenge in diabetic patients. Regulating blood sugar by limiting glucose uptake in the intestines and promoting its utilization in tissues offers an effective way to manage this issue. Natural compounds derived from plants are increasingly investigated for their ability to inhibit enzymes like tyrosinase and cholinesterases, offering safer and potentially more effective solutions (Eruygur & Uçar, 2018; Thilagam *et al*., 2013).

The *Sideritis* genus, a key group within the Lamiaceae family (subfamily Lamioideae), consists of approximately 150 species, primarily distributed across the Eastern and Western Mediterranean regions (de Castro & Rivera Núñez, 1994). Commonly referred to as "mountain tea," these plants are traditionally consumed as infusions with tonic, carminative, diuretic, and digestive properties (Özcan *et al*., 2001). Additionally, they are highly regarded as culinary herbs and for their use in flavor production (Palá-Paúl *et al*., 2006). In traditional medicine, *Sideritis* species are extensively employed for their anti-inflammatory, anti-ulcer, antimicrobial, cytostatic, astringent, and vulnerary effects, as well as for treating flu and stimulating circulation (Palomino *et al*., 1996). The genus is notable for its secondary metabolites, particularly diterpenoids with an ent-kaurane structure, which exhibit various bioactivities (Alessandro Venditti *et al*., 2016). Essential oils from *Sideritis* species can be categorized into three primary chemotypes: monoterpene-rich, sesquiterpene-rich, and diterpene-rich (Giuliani *et al*., 2011; Kirimer *et al*., 2004). Other bioactive components include flavonoids, phenylpropanoid and phenylethanoid glycosides, and iridoids (Alessandro Venditti *et al*., 2016; Venditti *et al*., 2013).

This study aims to comprehensively evaluate the chemical composition, antioxidant potential, and enzyme inhibitory activities of the methanol extract obtained from *Sideritis montana* L. subsp. *montana* using ultrasound-assisted extraction. By quantifying the total phenolic and flavonoid contents and identifying the key phytochemicals through advanced chromatographic techniques, we seek to elucidate the bioactive components responsible for the plant's biological properties. Additionally, the investigation of antioxidant and enzyme inhibitory activities will provide critical insights into the therapeutic potential of this subspecies. This research not only contributes to the growing body of knowledge on *Sideritis* species but also highlights the importance of innovative extraction techniques in enhancing the discovery of natural compounds with promising pharmacological applications.

2. MATERIAL and METHODS

2.1. Plant Material

Aerial parts of *S. montana* subsp. *montana* were harvested on May 17, 2022, from Nebiler village, Kavaklıdere-Muğla, Türkiye (elevation: 1060 m, coordinates: 37°27'35"N, 28°24'60"E). The species was identified by Dr. Olcay Ceylan, and a voucher specimen (O.2154) was deposited in the Herbarium of the Department of Biology, Muğla Sıtkı Koçman University, Türkiye. The collected material was air-dried in shaded conditions for several weeks and ground into a fine powder using a laboratory grinder.

2.2. Methanol Extraction

Ultrasound-assisted extraction (UAE) was employed to prepare the methanol extract, using a sample-to-solvent ratio of 1:20. The process was performed in a sonication bath for one hour. After extraction, the methanol was removed under reduced pressure with a rotary evaporator, and the obtained extract was stored at 4°C for further analysis.

2.3. Determination of Chemical Composition

The total flavonoid content (TFC) was quantified using the aluminum chloride method, while the total phenolic content (TPC) was assessed with Folin-Ciocalteu reagent. Results were expressed as rutin equivalents (REs) and gallic acid equivalents (GAEs), respectively, following protocols described by Sarikurkcu *et al*. (2013). The phytochemical composition was further analyzed using a validated chromatographic method reported by Cittan and Çelik (2018). Detailed methodological parameters are provided in the supplementary material.

2.4. Biological Activity

Antioxidant activity was evaluated using multiple methods (Apak *et al*., 2006; Kocak *et al*., 2016; Sarikurkcu *et al*., 2020; Tepe *et al*., 2011; Zengin *et al*., 2017;), while enzyme inhibitory activities were assessed based on the protocols of Ozer *et al*. (2018). Full descriptions of the methods are presented in the supplementary file.

2.5. Statistical Analysis

All results are expressed as means ± SD, based on three independent replicates)

3. RESULTS and DISCUSSION

3.1. Chemical Composition

The methanol extract of *S. montana* subsp. *montana*, obtained using ultrasound-assisted extraction, exhibited an extraction yield of 5.37%. The total phenolic content of the extract was determined to be 63.27 mg GAEs/g extract, while the total flavonoid content was measured as 58.32 mg REs/g extract (Table 1). These findings indicate that the extract possesses a relatively high concentration of phenolic and flavonoid compounds, which may contribute to its potential bioactivity. All values represent the mean ± standard deviation (SD) of three independent replicates, with phenolic and flavonoid contents expressed in terms of gallic acid and rutin equivalents, respectively.

Table 1. Extraction yield, total phenolic and flavonoid contents of the methanol extract from *S. montana* subsp. *montana*.

Assays	Methanol extract
Extraction yield (%)	5.37
Total flavonoids (mg REs/g extract)	58.32±0.05
Total phenolics (mg GAEs/g extract)	63.27±2.17

Values expressed are means ± SD of three parallel measurements. REs and GAEs rutin and gallic acid equivalents.

The chemical composition analysis of the methanol extract from *S. montana* subsp. *montana*, obtained through ultrasound-assisted extraction, revealed the presence of various phenolic

compounds in differing concentrations (Table 2). Among the quantified compounds, chlorogenic acid was identified as the most abundant (563 µg/g extract), followed by luteolin 7-glucoside (513 µg/g extract) and hyperoside (511 µg/g extract). Moderate levels of luteolin (41.1 µg/g extract), 4-hydroxybenzoic acid (40.4 µg/g extract), and 3-hydroxybenzoic acid (39.3 µg/g extract) were also detected. Additionally, apigenin 7-glucoside, *p*-coumaric acid, and vanillin were present in concentrations of 39.3, 33.0, and 20.1 µg/g extract, respectively.

Other phenolic compounds, such as verbascoside, 2,5-dihydroxybenzoic acid, and protocatechuic acid, were identified in smaller amounts. Flavonoids like and apigenin were also detected, albeit at lower concentrations. Compounds such as ferulic acid, pinoresinol, and syringic acid were present in trace amounts. In contrast, certain compounds, including 3,4-dihydroxyphenylacetic acid, 2-hydroxycinnamic acid, and rosmarinic acid, were not detected in the extract.

The chemical composition of the methanol extract obtained via ultrasound-assisted extraction from *S. montana* subsp. *montana* presents novel and significant findings when compared to the previously reported data in the literature.

Table 2. Concentration of selected phenolic compounds in the methanol extract from *S. montana* subsp. *montana*.

Compounds	Concentration (µg/g extract)
Chlorogenic acid	563±25
Luteolin 7-glucoside	513±6
Hyperoside	511±3
Luteolin	41.1±0.7
4-Hydroxybenzoic acid	40.4±0.5
3-Hydroxybenzoic acid	39.3±1.7
Apigenin 7-glucoside	39.3±3.6
p-Coumaric acid	33.0±0.3
Vanillin	20.1±3.2
Verbascoside	18.4±0.1
2,5-Dihydroxybenzoic acid	18.2±0.3
Protocatechuic acid	17.2±0.6
Quercetin	13.8±0.1
Apigenin	11.0±0.3
Ferulic acid	9.54±0.45
Pinoresinol	6.84±0.33
Caffeic acid	4.82±0.74
Syringic acid	5.26±0.02
Hesperidin	4.88±0.32
Gallic acid	1.74±0.01
Eriodictyol	0.56±0.03
(-)-Epicatechin	0.22±0.01
3,4-Dihydroxyphenylacetic acid	nd
2-Hydroxycinnamic acid	nd
(+)-Catechin	nd
Pyrocatechol	nd
Sinapic acid	nd
Taxifolin	nd
Rosmarinic acid	nd
Kaempferol	nd

Values expressed are means ± SD of three parallel measurements.

The current study reports a total phenolic content and a total flavonoid content, indicative of high phenolic and flavonoid concentrations. These values surpass the phenolic and flavonoid content observed in earlier studies that focus on ethanolic or seed extracts of *S. montana* subsp. *montana* (e.g., Emre *et al*. (2011)). This discrepancy may stem from differences in extraction techniques and solvent systems, as ultrasound-assisted extraction is known to enhance the recovery of bioactive compounds.

The current analysis identified chlorogenic acid, luteolin 7-glucoside, and hyperoside as the predominant compounds. While flavonoids and caffeoylquinic derivatives were previously noted in the work of A. Venditti *et al*. (2016), the quantified levels of these compounds, especially chlorogenic acid, are significantly higher in the present study. This may suggest a potential geographical or ecological variability influencing the plant's metabolomic profile.

In contrast to earlier studies that highlighted flavonoids such as morin and catechin as dominant (Emre *et al*., 2011), the current findings emphasize luteolin and its glycosides, hyperoside, and apigenin 7-glucoside as key flavonoids. These differences could be attributed to variations in plant part usage, solvent polarity, or specific environmental factors influencing secondary metabolite biosynthesis.

The presence of 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, vanillin, and *p*-coumaric acid in the current study constitutes a new record for *S. montana* subsp. *montana*. These phenolic acids and aromatic compounds have not been previously reported for this subspecies in the literature, highlighting their novelty as phytochemical constituents. These findings contribute to the chemotaxonomic characterization of the species and broaden the phytochemical spectrum associated with the genus *Sideritis*.

The predominance of chlorogenic acid and specific flavonoids aligns with the chemotaxonomic markers of the *Sideritis* genus, as discussed by Kilic (2014). However, the identification of unique phenolic acids and aromatic compounds, such as vanillin and *p*-coumaric acid, differentiates *S. montana* subsp. *montana* from closely related taxa, supporting its distinct chemotaxonomic status.

The novel compounds identified in this study, combined with the high phenolic and flavonoid content, underscore the bioactive potential of *S. montana* subsp. *montana*. The presence of compounds like vanillin and *p*-coumaric acid, which are known for their antioxidant and antimicrobial properties, suggests potential applications in pharmaceutical and nutraceutical formulations.

The current findings not only align with the general chemical characteristics of *Sideritis* species but also extend the phytochemical knowledge of *S. montana* subsp. *montana* by reporting new compounds and higher concentrations of known constituents. These results underscore the importance of using advanced extraction and analysis methods for uncovering the full metabolomic potential of medicinal plants.

3.2. Antioxidant Activity

The antioxidant potential of the methanol extract from *S. montana* subsp. *montana*, obtained via ultrasound-assisted extraction, was evaluated using multiple in vitro assays, with results indicating notable activity across various mechanisms (Table 3). The highest antioxidant activity was observed in the phosphomolybdenum assay, with a total antioxidant capacity of 428.52 mg TEs/g extract, reflecting the extract's strong ability to reduce molybdenum(VI) to molybdenum(V). This was followed by significant activity in the CUPRAC (217.40 mg TEs/g extract) and FRAP (171.33 mg TEs/g extract) assays, both of which assess reducing power and suggest the presence of compounds capable of donating electrons to neutralize reactive species. The radical scavenging activities, evaluated using DPPH and ABTS assays, were measured at 122.76 and 140.41 mg TEs/g extract, respectively, indicating the extract's moderate efficiency in neutralizing free radicals. In contrast, the ferrous ion chelating activity was relatively low, at

6.62 mg EDTAEs/g extract, suggesting a limited ability to bind metal ions and inhibit metal-catalyzed oxidative reactions.

Table 3. Antioxidant activity of the methanol extract from *S. montana* subsp. *montana*.

Assays	Activity
Phosphomolybdenum (mg TEs/ g extract)	428.52±7.81
CUPRAC reducing power (mg TEs/ g extract)	217.40±14.54
FRAP reducing power (mg TEs/ g extract)	171.33±2.13
DPPH radical scavenging (mg TEs/ g extract)	122.76±2.42
ABTS cation radical scavenging (mg TEs/ g extract)	140.41±6.69
Ferrous ion chelating (mg EDTAEs/ g extract)	6.62±0.59

Values expressed are means ± SD of three parallel measurements. TEs and EDTAEs, trolox and ethylenediaminetetraacetic acid (disodium salt) equivalents, respectively.

When the antioxidant activity results are considered alongside the chemical composition data, the high activity observed in the phosphomolybdenum, CUPRAC, and FRAP assays can be attributed to the extract's abundant phenolic and flavonoid content, particularly chlorogenic acid, luteolin 7-glucoside, and hyperoside. These compounds are well-documented for their electron-donating properties, which likely contribute to the extract's robust reducing power and overall antioxidant capacity (Gao *et al*., 2019; Maatouk *et al*., 2017; Park *et al*., 2016; Sato *et al*., 2011; Wu, 2007). The moderate radical scavenging activities observed in the DPPH and ABTS assays may also be linked to these phenolics, as well as other bioactive constituents such as luteolin and *p*-coumaric acid, which are known to quench free radicals through hydrogen atom transfer or single electron transfer mechanisms (Gökbulut *et al*., 2012; Kiliç & Yeşiloğlu, 2013; Masek *et al*., 2016; Tian *et al*., 2021).

The relatively low ferrous ion chelating activity suggests that metal chelation is not a primary mechanism of action for this extract, which aligns with the chemical composition data indicating lower concentrations of compounds like quercetin and apigenin, which possess chelating properties. Overall, the methanol extract's antioxidant profile underscores the prominent role of its phenolic constituents, particularly chlorogenic acid, luteolin 7-glucoside, and hyperoside, in contributing to its bioactivity.

3.3 Enzyme Inhibitory Activity

The enzyme inhibition potential of the methanol extract from *S. montana* subsp. *montana* was evaluated through multiple assays targeting key enzymes involved in various physiological and pathological processes (Table 4).

The extract demonstrated the highest inhibitory activity against α-glucosidase, with a value of 753.81 mg ACEs/g extract. This suggests a strong potential for managing hyperglycemia through the modulation of carbohydrate metabolism. Similarly, significant inhibition was observed for α-amylase, with an activity of 274.95 mg ACEs/g extract, indicating its complementary role in reducing postprandial glucose levels.

Table 4. Enzyme inhibition activity of the methanol extract from *S. montana* subsp. *montana*.

Assays	Activity
Acetylcholinesterase inhibition (mg GALAEs/g extract)	2.08±0.03
Butyrylcholinesterase inhibition (mg GALAEs/g extract)	0.45±0.01
Tyrosinase Inhibition (mg KAEs/g extract)	68.56±0.26
α-Amylase inhibition (mg ACEs/g extract)	274.95±3.06
α-Glucosidase inhibition (mg ACEs/g extract)	753.81±8.87

Values expressed are means ± SD of three parallel measurements. ACEs, GALAEs and KAEs mean acarbose, galanthamine and kojic acid equivalents, respectively.

The tyrosinase inhibition assay revealed moderate activity, with a value of 68.56 mg KAEs/g extract, suggesting potential applicability in dermatological formulations targeting hyperpigmentation. In contrast, the extract exhibited relatively low inhibition against acetylcholinesterase (2.08 mg GALAEs/g extract) and butyrylcholinesterase (0.45 mg GALAEs/g extract), indicating limited efficacy in addressing cholinesterase-associated disorders, such as Alzheimer's disease.

When these findings are considered alongside the chemical composition data, the observed enzyme inhibitory activities can be attributed to the high phenolic and flavonoid content of the extract. The strong α-glucosidase and α-amylase inhibitory activities may be linked to the abundance of chlorogenic acid, which has been widely reported to inhibit carbohydrate-hydrolyzing enzymes (Oboh *et al*., 2015; Wang *et al*., 2022; Zheng *et al*., 2020). Additionally, compounds such as hyperoside (Shen *et al*., 2023), and *p*-coumaric acid (Huang *et al*., 2023; Khan *et al*., 2022) may contribute to these effects through their known enzyme-modulating properties.

The moderate tyrosinase inhibition activity is likely influenced by phenolics such as vanillin (Ashraf *et al*., 2015; Rescigno *et al*., 2011) and apigenin 7-glucoside (Bouzaiene *et al*., 2016; Sezen Karaoğlan *et al*., 2023), both of which are recognized for their ability to interfere with melanin biosynthesis. The relatively weak cholinesterase inhibition observed in the study may reflect the low concentrations of specific compounds, such as quercetin and apigenin, that are more commonly associated with acetylcholinesterase and butyrylcholinesterase inhibition (Islam *et al*., 2021; Orhan, 2021).

When combined with the previously reported antioxidant activity of the extract, these results suggest that the bioactive potential of *S. montana* subsp. *montana* methanol extract arises from the synergistic effects of its phenolic constituents. The pronounced enzyme inhibition against α-glucosidase and α-amylase highlights its potential for managing oxidative stress and metabolic disorders, particularly diabetes. The contributions of chlorogenic acid, luteolin derivatives, and other phenolics underscore the importance of these compounds in defining the extract's biofunctional properties.

4. CONCLUSION

The findings of this study provide substantial evidence for the bioactive potential of the methanol extract derived from *S. montana* subsp. *montana* through ultrasound-assisted extraction. The extract’s high content of phenolic and flavonoid compounds underscores its relevance as a source of antioxidant and enzyme-inhibitory agents. Among the phenolic constituents, chlorogenic acid, luteolin 7-glucoside, and hyperoside were identified as the predominant compounds, which likely play a critical role in the observed bioactivities.

The extract exhibited pronounced antioxidant activity, particularly in the phosphomolybdenum assay, demonstrating its capacity to neutralize reactive oxygen species. This was further supported by notable CUPRAC and FRAP reducing power, which reflect the presence of potent electron-donating molecules. Moderate radical scavenging activity in the DPPH and ABTS assays and limited ferrous ion chelating ability suggest a multifaceted antioxidant mechanism, where electron transfer predominates over metal chelation. The chemical composition analysis reinforces these results, as the identified phenolics, especially chlorogenic acid and flavonoid derivatives, are well-documented for their antioxidant properties.

In terms of enzyme inhibition, the extract showed significant activity against carbohydrate-hydrolyzing enzymes, particularly α-glucosidase and α-amylase, indicating strong potential for managing hyperglycemia and diabetes-related oxidative stress. Moderate tyrosinase inhibition suggests additional applications in dermatology, particularly for hyperpigmentation disorders. However, the extract demonstrated limited cholinesterase inhibitory activity, which may diminish its therapeutic applicability in neurodegenerative diseases like Alzheimer's.

Despite these promising results, certain limitations should be acknowledged. While the study

highlights the extract's bioactive potential, the exact synergistic or individual contributions of the phenolic compounds remain unclear. Advanced studies, such as bioactivity-guided fractionation or molecular docking, could elucidate the mechanisms underlying these effects. Additionally, in vivo investigations are necessary to validate the efficacy and safety of the extract under physiological conditions, as in vitro assays may not fully represent its behavior in complex biological systems.

Future research should also explore the optimization of extraction parameters to further enhance the yield and activity of bioactive compounds. Comparative studies with other extraction techniques and solvents could provide insights into the efficiency and sustainability of ultrasound-assisted extraction for *S. montana* subsp. *montana*. Finally, assessing the stability of the bioactive components under storage and processing conditions would be critical for potential industrial or pharmaceutical applications.

In conclusion, the methanol extract of *S. montana* subsp. *montana* holds significant promise as a source of natural antioxidants and enzyme inhibitors. Its chemical richness and multifunctional bioactivities highlight its potential for therapeutic and nutraceutical applications, warranting further exploration to fully realize its benefits.

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Authorship Contribution Statement

Fatma Özlem Kargın Solmaz contributed to the experimental studies, performed antioxidant and enzyme inhibition activity experiments, preparation of manuscript and proofreading; **Cengiz Sarikurkcu** provided the plant material used, participated in phytochemical analysis and the writing and proofreading.

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Research Article

Antimicrobial activity, phytochemical characterization and molecular docking studies of *Nyctanthes arbor-tristis* L. extracts

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Abstract: Antimicrobial resistance among nosocomial pathogens reduces the efficacy of antibiotics and lead to treatment failure among susceptible patients, which necessitates the identification of novel antimicrobial agents. *Nyctanthes arbor-tristis* L. is a valuable medicinal plant with numerous bioactive phytochemicals, which could be explored for their antimicrobial potential. This study evaluated the antimicrobial activity of hexane fractions of ethanolic extracts of *Nyctanthes arbor-tristis* against nosocomial bacteria *Escherichia coli, Bacillus subtilis, Pseudomonas florescens, Aeromonas hydrophila, Enterococcus faecalis* and *Kleibsella pneumonia*, determined the phytochemical composition and predicted potential antimicrobial compounds through in-silico method. The hexane fractions of ethanolic extracts of *Nyctanthes arbor-tristis* were obtained by maceration and solvent partitioning, and further characterized through gas chromatography–mass spectrometry. The hexane fractions were examined in-vitro for antibacterial activity by the disc diffusion method and minimal inhibitory concentration (MIC) was determined on the basis of optical density. Molecular docking was done using AutoDockTools 1.5.7 and Pyrx. The leaf and stem samples exhibited significant antimicrobial activity against *E. coli, B. subtilis, P. florescens* and *A. hydrophilla*. The major compounds identified through GC-MS phytol, 1,2-benzenedicarboxylic acid and dioctyl phthalate were docked; the docking scores were -6.4, -6.6 and -7.2 respectively against 6KVP, while -6.2, -6.7 and -7.6 respectively against 4FS3. This study gives the first report of the antimicrobial activity of non-polar fractions of ethanolic extracts of *Nyctanthes arbor-tristis* against nosocomial bacteria and lead to the identification of phytol, 1,2-benzenedicarboxylic acid and dioctyl phthalate as novel potential antimicrobial agents.

1. INTRODUCTION

Nosocomial infections are health-care-associated infections acquired by the patients in the hospital during their course of stay, which constitute added burden on the healthcare system. Commonly encountered nosocomial bacterial infections are ventilator-associated pneumonia, catheter-associated urinary tract infections, central line-associated bloodstream infections, and surgical site related skin and soft-tissue infections (Agaba *et al.*, 2017). Patients in the hospital are predisposed to nosocomial infections due to factors like transmission of pathogen, immune suppression, monotherapy or inadequate antibiotic treatment (O'Toole, 2021). Bacteria often

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develop new mechanisms to evade the antibiotic treatment, thus indiscriminate and overuse of antibiotics result in their ineffectiveness against the infections. Development of antimicrobial resistance (AMR) among nosocomial bacterial strains causes reduced efficacy of antibiotics and prolonged treatment, and also lead to increased mortality in highly susceptible patients due to treatment failure (Avershina *et al*., 2021). This necessitates the identification of novel antimicrobial agents which are effective against antimicrobial resistant nosocomial bacteria.

Phytochemicals obtained from medicinal plants have proven to be of immense therapeutic value and have been used as potential source of antimicrobial and bioactive compounds (Wikaningtyas & Sukandar, 2016; Vijayalakshmi & Shourie, 2019). Computational approaches for prediction of bioactivity of phytochemicals are highly useful in finding efficient drug candidates against AMR bacterial pathogens. Molecular docking gives valuable predictive information regarding molecular interactions between phytochemical ligand and receptor protein, deciphering the mechanism of antimicrobial activity (Mir *et al*., 2022).

Nyctanthes arbor-tristis also named as Parijat is a member of the family Oleaceae and a valuable medicinal plant distributed in India and several other parts of Asia. It has been used in many traditional and alternative medicine systems such as Ayurveda, Unani, Siddha and Chinese medicine. It is a rich source of bioactive phytochemicals with many pharmacological benefits such as they act as anti-inflammatory compounds, relieve joint pain and rheumatic arthritis, exhibit antioxidant and hepato-protective activity, and also show anti-helminthic and anti-pyretic action (Karan *et al*., 2019; Sharma & Shourie, 2023). *Nyctanthes arbor-tristis* extracts have been shown to possess significant antimicrobial activities, while most of the studies have investigated its polar extracts using solvents like methanol, ethanol, dichloromethane, chloroform and ethyl acetate (Khatune *et al*., 2001, Singh & Solanki, 2022). However, the non-polar extracts of *Nyctanthes arbor-tristis* also possess a wide array of phytochemicals which could be explored for antimicrobial activity.

This study aimed at evaluation of hexane fractions of ethanolic extracts of leaf, stem and flower of *Nyctanthes arbor-tristis* for their antimicrobial activity against common nosocomial pathogens *Escherichia coli, Bacillus subtilis, Pseudomonas florescens, Aeromonas hydrophila, Enterococcus faecalis* and *Kleibsella pneumoniae*, which are known to develop antimicrobial drug resistance and potentially contribute to therapeutic failure. A broad phytochemical characterization of these extracts was also undertaken in order to identify the potential antimicrobial drug lead compounds and the mechanisms behind the antimicrobial activity exhibited by the *Nyctanthes arbor-tristis* extracts were elucidated by in-silico molecular docking.

2. MATERIAL and METHODS

2.1 Chemicals and Reagents

Ethanol, hexane, dimethyl sulfoxide (DMSO) and resazurin dye were purchased from Himedia, New Delhi, India. The antibiotic Ampicillin was obtained from SRL (Sisco Research Laboratories), New Delhi, India. Nutrient broth and Mueller Hinton Agar was procured from Titan Biotech, New Delhi, India.

2.2 Collection and Identification of Plant Material

Nyctanthes arbor-tristis plant parts were collected in the month of January, from Sector 54, Gurgaon (Coordinates N 28° 26' 38.2956", E 77° 6' 40.0356") and the flowering specimen of the plant were submitted for authentication to National Bureau of Plant Genetic Resources, National Herbarium of Cultivated Plants, Pusa Campus, New Delhi, India, and authentication certificate No, AC-29/2021was obtained.

2.3 Extraction and Chemical Characterization of the Phytochemicals

Plant material was shade dried, powdered mechanically and macerated in 70% ethanol (50 g / 500 mL) for 5 days. The crude extracts were concentrated on a rotary evaporator (Make-

Labtherm, Model- LT40, India) at 40ºC, re-dissolved in double distilled water and partitioned thrice with hexane using a separating funnel. The extraction and purification of phytochemicals was done according to the methods described by Harborne (1984). All hexane fractions were subjected to Gas chromatography mass spectroscopy (GC-MS) separately. The sample (1.0 µL) was injected into the gas chromatography system (GC-MS – QP 2010 Ultra Shimadzu) with 1:20 flow split. The isolation of constituents was conducted in Restek GC Column RXi® - 5Sil MS (Crossbond ®, similar to 5% diphenyl / 95% dimethylploysiloxane, 30m x 0.25mm x 0.25µm) with helium as the carrier gas (1.21 mL/min). Temperature programming was maintained from 100°C to 250°C with constant rise of 10°C/min and then held isothermal at 250°C for 10 min; further the temperature was increased by 15°C/min up to 280°C and again held isothermal. The electronic ionization mode of 70eV was maintained for the operation of the mass detector. For the identification, National Institute of Standards and Technology (NIST) spectral libraries were used to compare the mass spectra of the chemical constituents with their retention time (Wulandari *et al*., 2024).

2.4 Antimicrobial activity Testing

2.4.1 Microbial strains

The microorganisms used in antimicrobial testing were procured from The Microbial Type Culture Collection and Gene Bank (*MTCC*), Chandigarh, India. The standard bacterial strains used in the study were *Escherichia coli* MTCC 1652, *Bacillus subtilis* MTCC 5981, *Pseudomonas florescens* MTCC 6627, *Aeromonas hydrophila* MTCC 1739, *Klebsiella pneumoniae* MTCC 109 and *Enterococcus faecalis* MTCC 439.

2.4.2 Disc Diffusion Assay

Extracts were dissolved in DMSO to obtain the final concentration of 10 mg/mL, which were used for assessment of antimicrobial activity through disc diffusion method (20 µL per disc). The antibiotic Ampicillin (100 µg/mL concentration) was used as a positive control while 10% DMSO was used as a negative control (Mohamed *et al*., 2020).

2.4.3 Minimum inhibitory concentration (MIC)

MIC was determined by microdilution method in which 50 µL of the bacterial inoculum was added to all the wells of the microtitre plate followed by addition of 20 µL hexane extracts in the concentration ranging from 1000 µg/mL to 0.488 µg/mL. The microdilution plates were placed in the incubator for 24h at 37°C, after adding 2 µL of resazurin dye (10 mg/mL) as colorimetric indicator and the optical density (OD) values were recorded (Mohamed *et al*., 2020).

2.5 Molecular Docking Studies

The major antimicrobial compounds identified through GC-MS in hexane fractions of ethanolic extracts of *Nyctanthes arbor-tristis* plant parts were subjected to molecular docking with proteins that are vital for the existence of the bacteria under the study. The target proteins associated with the bacteria were selected for docking against the phytochemical ligands on the basis of literature. RCSB PUB CHEM (Research Collaboratory for Structural Bioinformatics PubChem database) was used to save the SDF files of the ligands, while Protein Data Bank (PDB) was used to save the PDB files of all the receptors proteins. AutoDockTools 1.5.7 was used to save the PDBQT files of these receptor proteins, and Pyrex was used to conduct the docking and ascertain the binding affinities (Khanum *et al*., 2024).

2.6 Statistical Analysis

All experiments concerning antimicrobial activity by disc diffusion method were performed in triplicate and the results have been represented as mean ± SD (standard deviation). Means were subjected to one-way analysis of variance (ANOVA) and the mean comparisons were performed by Tukey's Honest Significant Difference (HSD) test at a significance level of $p<0.01$ using SPSS (Statistical Package for Social Sciences) version 20.0.

3. RESULTS and DISCUSSION

3.1 Antimicrobial Activity

3.1.1 Disc Diffusion Assay

The hexane fractions of ethanolic extracts of leaf (NAT-ETHL) and stem (NAT-ETHS) of *Nyctanthes arbor-tristis* exhibited remarkable antimicrobial activity against *E. coli, B. subtilis, P. florescens, A. hydrophilla* and *E. faecalis*. The hexane fractions of ethanolic extracts of flower (NAT-ETHF) was very less active against *E. coli, B. subtilis, P. florescens* and *K. pneumoniae*, whereas it did not inhibit the growth of *A. hydrophilla* and *E. faecalis* at all. The activity of all the samples was compared to the zone of inhibition formed by standard antibiotic Ampicillin used as positive control (PC) against all the bacteria, while DMSO was used as a negative control. The zone of inhibition (diameter significant at $p<0.01$) formed by NAT-ETHL were against *E. coli, B. subtilis* and *P. florescens* measured as 12.2 ± 0.30 mm, 9.6 ± 0.95 mm and 14.5 ± 0.40 mm respectively, while those formed by NAT-ETHS were against *B. subtilis, P. florescens* and *A. hydrophilla* measured as 16.3 ± 0.30 mm, 17.6 ± 0.40 mm and 16.4 ± 0.27 mm respectively (Table 1).

Table 1. Antimicrobial activity of hexane fractions of ethanolic extracts of *Nyctanthes arbor-tristis* plant parts against nosocomial bacteria.

Samples	*Escherichia coli*	*Bacillus subtilis*	*Pseudomonas florescens*	*Aeromonas hydrophila*	*Enterococcus faecalis*	*Klebsiella pneumoniae*
ETHL	12.2 ± 0.30^{a}	9.6 ± 0.95^{a}	14.5 ± 0.40^{a}	13.6 ± 2.8^{abd}	13.8 ± 0.90^{ab}	1.3 ± 0.46^{ac}
ETHS	17.1 ± 1.85^{bd}	16.3 ± 0.30^{b}	17.6 ± 0.40^{b}	16.4 ± 0.27^{b}	13.2 ± 0.75^{bd}	N
ETHF	1.7 ± 0.50^{c}	1.5 ± 0.50^{c}	0.91 ± 0.01^{c}	N	N	1.2 ± 0.37^{c}
Ampicillin	21.2 ± 1.30^{d}	23.7 ± 1.7^{d}	22.2 ± 0.68^{d}	23.3 ± 0.65^{d}	14.6 ± 1.35^{d}	12.2 ± 0.30^{d}
DMSO	N	N	N	N	N	N

Values are means of triplicate determination (n=3) ± standard deviations. Means followed by the same superscript letter(s) in the same column are not significantly different at $p \leq 0.01$ according to the post hoc Tukey's HSD test. N denotes no zone of inhibition.

Dichloromethane and ethyl acetate extracts of *Nyctanthes arbor-tristis* flower have been found to show significant antimicrobial activity against *Bacillus cereus, B. subtilis, Staphylococcus aureus* and *Pseudomonas sp*. (Khatune *et al*., 2001). The ethanolic leaf extracts of *Nyctanthes arbor-tristis* showed significant antimicrobial activity against *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Salmonella typhi* (Singh & Solanki, 2022; Khanam & Dwivedi, 2022). The *n*-hexane extracts of *Nigella sativa* showed pronounced antimicrobial activity against pathogenic bacteria *S. aureus* (inhibition zone diameter upto 8.35mm, MIC=1.28 mg/mL), *E. coli* (inhibition zone diameter upto 11.33 ± 0.85 mm, MIC 32 mg/mL) and *Streptococcus pyogenes* (inhibition zone diameter upto 15.35 mm, MIC 1.28 mg/mL) (Abraham *et al*., 2019). Hexane extract of *Ficus carica* L. were reported to exhibit remarkable antibacterial activity against *Staphylococcus saprophyticus* and *Staphylococcus aureus* (MIC = 19 µg/mL) and the compositional analysis of the extract revealed the presence of 36 compounds which were majorly coumarins (Lazreg-Aref *et al*., 2012).

3.1.2 Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MIC) of most bioactive samples NAT-ETHL and NAT-ETHS were determined through microdilution tests. MIC of NAT-ETHL was found to be 15.62 µg/mL against *E. coli, B. subtilis, A. hydrophila,* and 31.25 µg/mL against *P. florescens*. NAT-ETHS exhibited MIC of 15.62 µg/mL against *E. coli,* and 31.25 µg/mL against *B. subtilis, P. florescens* and *A. hydrophila*. MIC of both NAT-ETHL and NAT-ETHS against *E. faecalis* was found to be higher i.e. 125 µg/mL (Table 2). Hexane extracts of *Ficus congensis* showed MIC

of 8 mg/mL and 5 mg/mL respectively against *E. coli* and *B. subtilis* (Alaribe *et al*., 2011). Hexane fractions of *Nigella sativa* L. showed potent antifungal activity against *Candida albicans* at MIC = 8 µg/mL (Tiji *et al*., 2021). The hexane leaf extracts of *Anisopus mannii* exhibited remarkable antibacterial activity against human pathogenic bacteria *S. aureus* (MIC= 1.25 mg/mL, Minimum bactericidal concentration i.e. MBC= 5 mg/mL), *P. aeruginosa* (MIC= 1.25 mg/mL, MBC= 5 mg/mL) and *S. pyogenes* (MIC= 0.625 mg/mL, MBC= 2.5 mg/mL) (Musa *et al*., 2015).

The MIC of standard ampicillin was recorded as 4 µg/mL against bovine intrauterine *E. coli*, and ≤0.25 µg/mL in several *Bacillus* species such as *B. cereus, B. paralicheniformis* and *B. subtilis* (de Boer *et al*., 2015; Zhai *et al*., 2023). While studying the antibiotic resistance patterns of *Pseudomonas* spp. isolated from bulk-tank milk, 59.3 % of isolates were found to exhibit MIC value ≥256 µg/mL for ampicillin (Meng *et al*. 2020). MIC_{90} values of ampicillin ranged from 0.5 to 2 µg/mL against *E. faecalis*, while MIC_{90} and MIC_{50} were recorded as >64 µg/mL and >32 µg/mL respectively against *Aeromonas hydrophila* (Conceição *et al*., 2012; Mahmood *et al*., 2024).

Table 2. MIC of hexane fractions of ethanolic extracts of *Nyctanthes arbor-tristis* plant parts against nosocomial bacteria.

Samples	*Escherichia coli* (µg/mL)	*Bacillus subtilis* (µg/mL)	*Pseudomonas florescens* (µg/mL)	*Aeromonas hydrophila* (µg/mL)	*Enterococcus faecalis* (µg/mL)
ETHL	15.62	15.62	31.25	15.62	125.00
ETHS	15.62	31.25	31.25	31.25	125.00
ETHF	-	-	-	-	-

3.2 Phytochemical Profiling through GC-MS

The GC-MS profile of NAT-ETHL exhibited 17 peaks, each with distinct retention time. The composition represented prominent presence of phytol (37.58%) and 1,2-benzenedicarboxylic acid (16.02%). The other major compounds present in the sample were (Z)6,(Z)9-pentadecadien-1-ol (8.25%), undec-10-ynoic acid, undec-2-en-1-yl ester (7.09%), methyl linolenate (5.33%), hexadecanoic acid ethyl ester (4.20%) and cis-13-octadecenoic acid methyl ester (3.88%). The GCMS profile of NAT-ETHS showed 16 peaks at different retention times. The most abundant compound was dioctyl phthalate (80.36%), while other major compounds identified were 1,8,11-heptadecatriene, (Z,Z) (4.28%), glycidyl oleate (4.06%), glycidyl palmitate (1.96%), hexadecanoic acid ethyl ester (1.32%) and 9,12-octadecadienoic acid (Z,Z)-methyl ester (1.20%). The sample NAT-ETHF exhibited 18 compounds in the chromatogram, and the composition showed the major presence of dioctyl phthalate (69.80%). Other major compounds included phytol (5.82%), hexadecanoic acid ethyl ester (4.26%), trans,trans-9,12-octadecadienoic acid propyl ester (3.13%), 1,8,11-hepatadecatriene (Z,Z) (2.54%), glycidyloleate (2.37%), stigmasta-5,22-ethyl dien-3-ol (2.25%), (9z, 12 z)-9,12-octadecadienoate (1.37%), 9-octadecenamide (1.30%) and glycidyl palmitate (1.21%) (Table 3).

Phytol extracted from *Leptadenia pyrotechnica* was reported to exhibit antimicrobial activity against *E. coli, C. albicans*, and *A. niger* with MIC_{50} value of 62.5 µg/mL, and *S. aureus* with MIC_{50} value >1000 µg/mL (Ghaneian *et al*., 2015). Phytol showed antimicrobial activity against *P. aeruginosa* by inducing oxidative stress response through generation of reactive oxygen species (ROS) in the cell (Lee *et al*., 2016). GC-MS enabled to identify 32 compounds in the hexane leaf extracts of *Anisopus mannii* of which the major compounds were hexadecanoic acid-ethyl ester was 34%, oxirane hexadecyl- was 11% and 9, 12, 15-ctadecatrienoic acid ethyl ester, (Z, Z, Z) was 9.6% (Musa *et al*., 2015). The compound (Z)6, (Z)9-Pentadecadien-1-ol, identified through GC-MS in alcoholic extract of *Psydrax dicoccos* also showed prominent presence in the chromatogram and has been reported to contribute to

the antifungal activity of methanolic plant extract (Umaiyambigai et. al., 2017). Other compounds reported for their antimicrobial properties are hexadecanoic acid and tetradecanal (Bittencourt *et al*., 2015; Shaaban *et al*., 2021). Hexadecanoic acid ethyl ester from *Arisaema flavum* (Forssk.) showed anticancer activity against MCF-7 cell lines with IC_{50} of 25 µM (Nisa *et al*., 2022). Antimicrobial activity of *Leonotis ocymifolia* extracts was also attributed to the presence of methyl linolenate, *n*-hexadecanoic acid, phytol (21.35 %), and octadecenoic acid methyl ester (Oyedeji-Amusa *et al*., 2024).

Table 3. Phytochemical **c**ompounds identified in GC-MS chromatogram of Hexane fractions of *Nyctanthes arbor-tristis* plant parts.

S. No.	Name of Compound	CAS No.*	Molecular Formula	Molecular Weight (g/mol)	Retention Time (min)	Peak Area %		
						ETHL	ETHS	ETHF
1	Tetradecanal	124-25-4	$C_{14}H_{28}O$	212.37	11.254	0.42	0.8	-
2	cis,cis,cis-7,10,13-Hexadecatrienal	56797-43-4	$C_{16}H_{26}O$	234.38	13.183	0.81	-	-
3	Hexadecanoic acid, methyl ester	112-39-0	$C_{17}H_{34}O_2$	270.5	13.45	1.59	0.81	0.45
4	Hexadecanoic acid, ethyl ester	628-97-7	$C_{18}H_{36}O_2$	284.5	14.11	4.2	1.32	4.26
5	(Z)6,(Z)9-Pentadecadien-1-ol	77899-11-7	$C_{15}H_{28}O$	224.38	14.57	8.25	-	-
6	Undec-10-ynoic acid, undec-2-en-1-yl ester	-	$C_{22}H_{38}O_2$	334.5	14.8	7.09	-	-
7	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	112-63-0	$C_{19}H_{34}O_2$	294.5	15.08	1.06	1.2	0.48
8	cis-13-Octadecenoic acid, methyl ester	13058-55-4	$C_{19}H_{36}O_2$	296.5	15.14	3.88	-	-
9	Phytol	150-86-7	$C_{20}H_{40}O$	296.5	15.25	39.1	1.29	5.82
10	Methyl Stearate	112-61-8	$C_{19}H_{38}O_2$	298.5	15.382	-	0.42	-
15	Glycidyl palmitate	7501-44-2	$C_{19}H_{36}O_3$	312.5	15.553	1.14	1.96	-
11	trans, trans-9,12-Octadecadienoic acid, propyl ester	64-17-5	$C_{21}H_{38}O_2$	322.5	15.69	-	0.54	3.13
12	Ethyl (9Z, 12Z)-9,12-Octadecadienoate	3443-82-1	$C_{21}H_{38}O_4$	354.5	15.756	-	-	1.37
13	Linolenate<methyl->	301-00-8	$C_{19}H_{32}O_2$	292.5	15.76	5.33	-	-
16	Stearate ethyl	643-22-1	$C_{55}H_{103}NO_{15}$	1018.4	15.982	-	-	0.81
14	Glycidyl palmitate	7501-44-2	$C_{19}H_{36}O_3$	312.5	16.91	1.96	0.52	1.21
17	Bis (2-ethylhexyl) Adipate	103-23-1	$C_{22}H_{42}O_4$	370.6	17.724	-	0.84	0.63
18	1,8,11-Heptadecatriene, (Z,Z)-	56134-03-3	$C_{17}H_{30}$	234.4	18.61	2	4.28	2.54
19	Glycidyl Oleate	5431-33-4	$C_{21}H_{38}O_3$	338.5	18.66	-	4.06	2.37
20	1,2-Benzenedicarboxylic acid	88-99-3	$C_8H_6O_4$	166.13	19.35	16.02	0.45	-
21	Dioctyl Phthalate	117-84-0	$C_{24}H_{38}O_4$	390.6	19.42	-	80.36	69.8
22	Phenyl Palmitate	24632-92-6	$C_{22}H_{36}O_2$	332.5	20.642	-	-	0.74
23	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	6422-86-2	$C_{24}H_{38}O_4$	390.6	21.28	2.86	0.62	0.49
24	9-Octadecenamide	3322-62-1	$C_{18}H_{35}NO$	281.5	21.67	-	0.53	1.3
25	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,	7200-26-2	$C_{30}H_{50}O$	426.7	23.033	-	-	0.74
26	Vitamin E	10191-41-0	$C_{29}H_{50}O_2$	430.7	25.723	1.66	-	-
27	Stigmasta-5,22-Dien-3-ol	83-48-7	$C_{29}H_{48}O$	412.7	27.98	-	-	2.25
28	Stigmast-5-en-3-ol, (3. beta.)-	83-46-5	$C_{29}H_{50}O$	414.7	29.25	2.63	-	1.61
Total Peak Area percentage						100	100	100

*CAS No - unique identification number, assigned by the Chemical Abstracts Service, US

3.3 Molecular Docking of Major Phytochemicals against Bacterial Protein Targets

The major compounds identified by GC-MS in *Nyctanthes arbor-tristis* samples that is Phytol (PUB CHEM ID 5280435), 1,2-benzene dicarboxylic acid (PUB CHEM ID 1017) and Dioctyl phthalate (PUB CHEM ID 8346) were selected for molecular docking against target bacterial proteins 6KVP and 4FS3. In order to understand the mechanism of docking, the significant amino acids of target proteins participating in the interactions with the selected ligands were studied by imaging the best pose of the docked structure. The binding of both the ligands occurred at FtsZ site of 6KVP, which is a novel and promising target for antimicrobial agents against AMR bacteria. FtsZ consists of a GTP-binding site and a GTPase activating site and forms a divisome complex (Z-ring) through a GTP-dependent polymerization process, which is essential for bacterial cell division in both in both Gram-positive and Gram-negative bacteria (Chai *et al*., 2022). The FabI site of 4FS3 is an enoyl-acyl carrier protein reductase which plays a crucial role in lipids and fatty acid biosynthesis, which are essential for the integrity of the bacterial cell membrane.

The binding affinities exhibited by phytol, 1,2-benzene dicarboxylic acid and dioctyl phthalate were -6.4, -6.6 and -7.2 respectively against 6KVP. Phytol interacted with 6KVP through amino acids Leucine (LEU-272) and Alanine (ALA- 275, ALA-279) with alkyl and pi-alkyl interactions, Phenylalanine (PHE-294) with pi-sigma, and Valine (VAL-207), LEU-270, Serine (SER-271), Glutamine (GLN-276), Threonine **(**THR-296) and Isoleucine (ILE-298) with van der Waals forces. Ligand 1,2-benzene dicarboxylic acid interacted with 6KVP through amino acids LEU-200 through pi-sigma and hydrogen bonds, LEU-209 through pi-alkyl and hydrogen bonds, Aspartic acid (ASP-199), VAL-203 and GLY-205 through hydrogen bonds, and Asparagine (ASN-208), GLY-295, THR-296, VAL-297 and ASN-263 through van der Waals forces. Dioctyl phthalate interacted with 6KVP through amino acids Methionine (MET-226), VAL-297, VAL-203, ILE-228, LEU-200, LEU-261, VAL-307 and ILE-311 with alkyl and pi-alkyl interactions, ASP-199 with pi-anion interaction, THR-309 and ASN-263 with hydrogen bonds and ILE-197, THR-265, GLN-192, GLY-196, LEU-209, MET-262 and VAL-310 with Van der Waals forces.

Phytol, 1,2-benzene dicarboxylic acid and dioctyl phthalate also showed good binding affinity with FabI site of 4FS3 protein with the docking affinity of -6.2, -6.7 and -7.6 respectively. Phytol interacted with the amino acids of 4FS3 which were LEU-102 with hydrogen bond, ILE-20, Alanine (ALA-95), Tyrosine (TYR-147, TYR-157), VAL-201 with alkyl and pi-alkyl interactions, and GLY-13, SER-19, ALA-21, SER-93, MET-99, ARG-103, ASN-156, LYS-164, PRO-192, ILE-193, PHE-204, ILE-207, THR-195, SER-197 with van er Waals forces. The amino acids of 4FS3 that significantly interacted with 1,2-benzene dicarboxylic acid were ILE-94 with pi-sigma interaction, VAL-67 with pi-alkyl interaction, THR-38, GLY-13 and ARG-40 with hydrogen bonding, SER-44, TYR-39, MET-12, ILE-65, ASP-66 and ILE-120 with van der Waals forces. Dioctyl phthalate interacted 4FS3 through amino acids TYR-147 with pi-pi stacking and alkyl interaction, VAL-154, VAL-201, TYR-157, ILE-207, PHE-204, ILE-20, ALA-95, ALA-190 and LEU-102 with alkyl and pi-alkyl interactions, SER-197 with hydrogen bond, GLY-13, ALA-15, SER-19, SER-93, GLN-155, ASN-156, PRO-192, GLY-191, ILE-193, THR-145, THR-146, MET-160, LYS-164, THR-195 and LEU-196 with Van der Waals forces (Figure 1, Table 4)

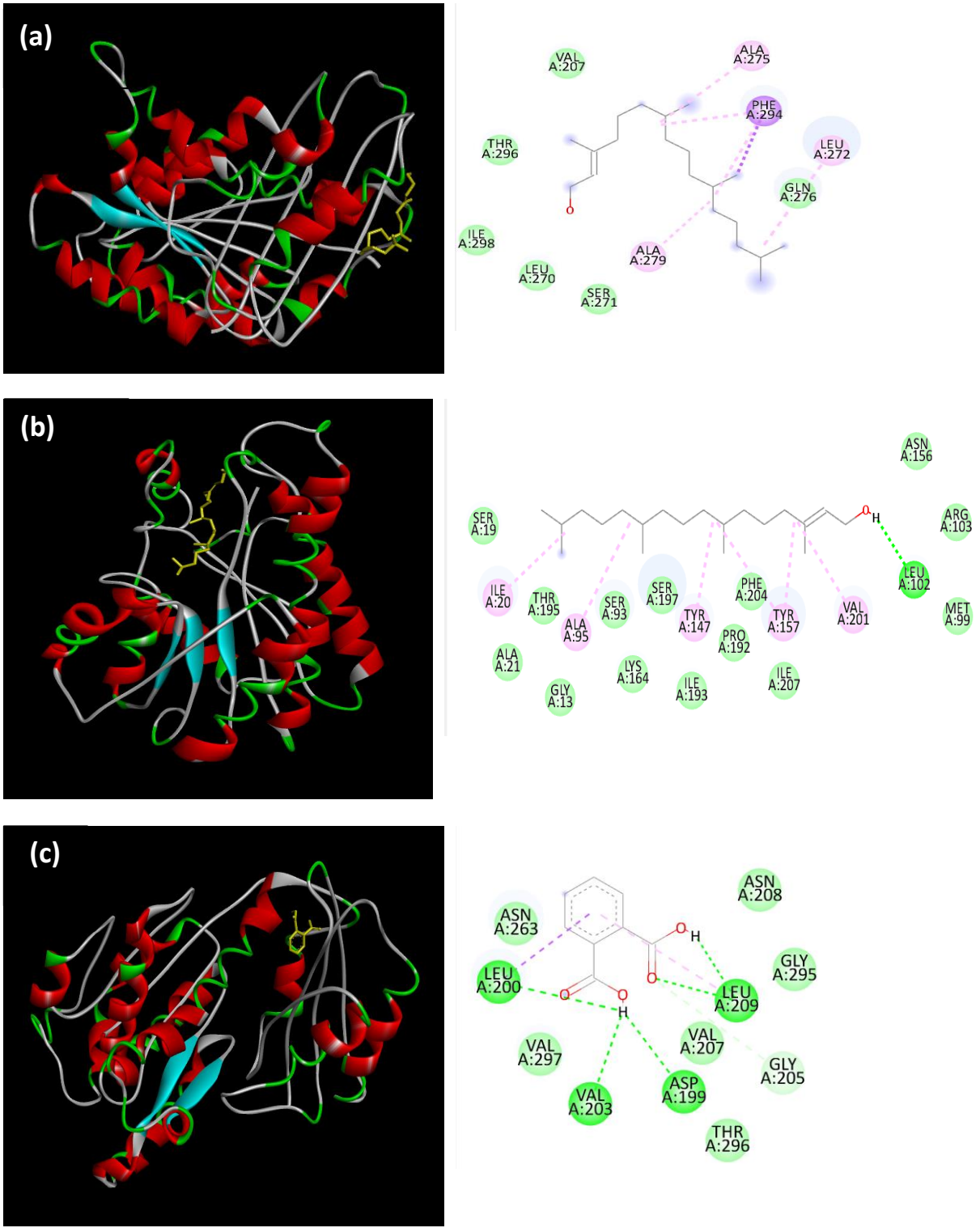

Figure 1. The 3D and 2D Molecular docking interactions major compounds identified by GC-MS in *Nyctanthes arbor-tristis* samples- (a) Phytol against 6KVP; (b) Phytol against 4FS3; (c) 1,2-benzene dicarboxylic acid against 6KVP; (d) 1,2-benzene dicarboxylic acid against 4FS3; (e) Dioctyl phthalate against 6KVP; (f) Dioctyl phthalate against 4FS3.

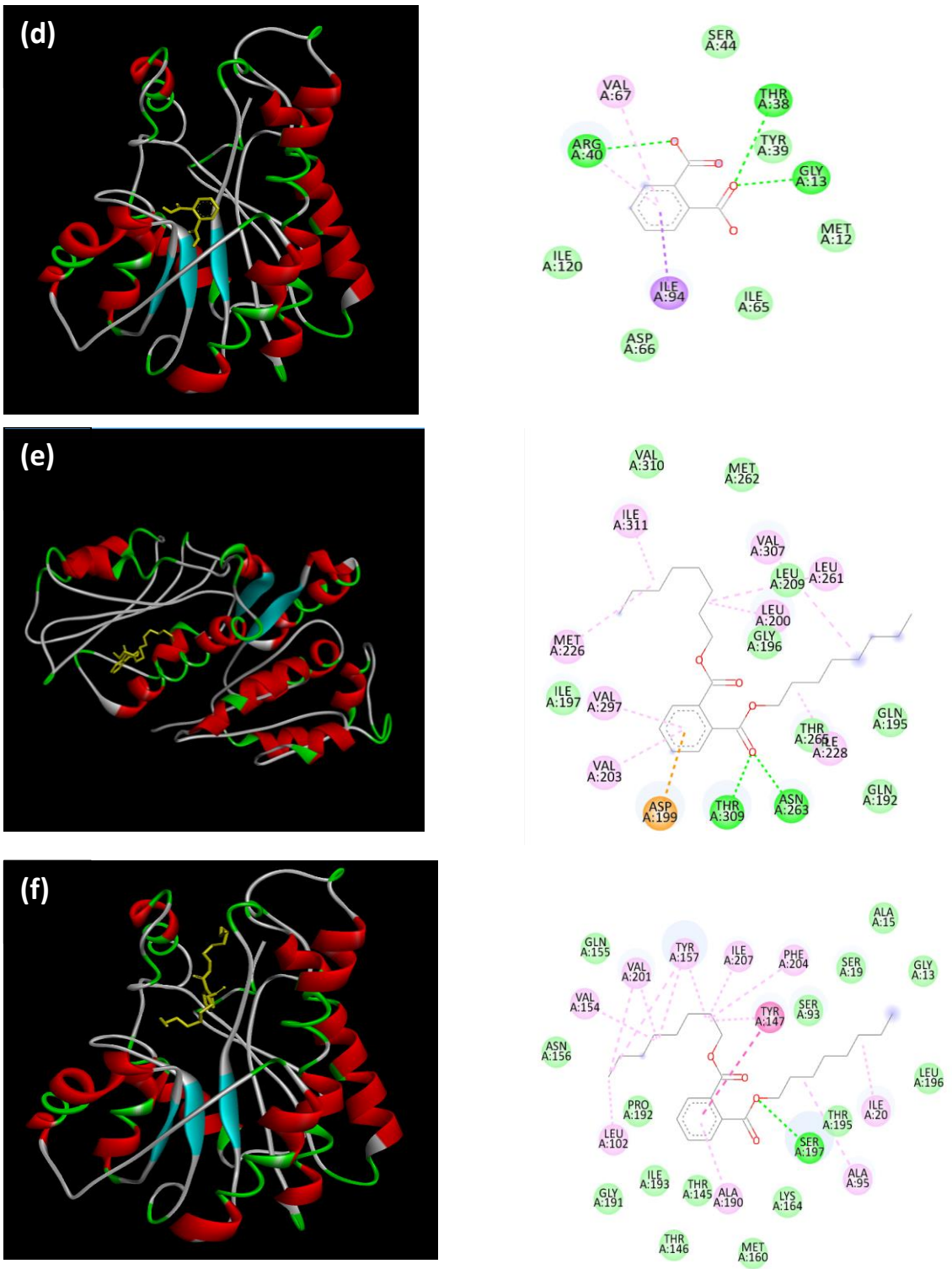

Figure 1. *Continues.*

Phytol has been found as a major compound in *Amaranthus lividus* extract and antimicrobial activity of phytol has been predicted through its molecular docking against bacterial proteins aquaporin-z, arginase and telomerase (Durhan *et al*., 2022). Benzene-1,3-dicarboxylic acid derivatives (furan based) exhibited effective inhibition of bacterial ligases MurC – MurF (Perdih *et al*., 2015). Another study corroborates the interaction of 1,2-benzene dicarboxylic acid with the pathogenicity sites and LpxC, a protein vital for bacterial survival (Rubab *et al*., 2018). Phthalates are used as drug coatings to facilitate localized drug release, mainly in gastro-intestinal drugs. Dibutyl phthalate has been docked against bacterial target protein AprX metalloprotease with a docking score of -5.8, for its significant antibacterial activity (Kumar *et al*., 2018).

Table 4. Molecular Docking Scores (binding affinities) and potential molecular interactions of phytol, 1,2-benzene dicarboxylic acid and Dioctyl phthalate with target bacterial proteins 6KVP and 4FS3.

S.No.	Target Bacterial Protein	Ligand	PUB CHEM ID	Free energy of binding (kcal/mol)	Amino acids interactions		
					alkyl/ pi-alkyl/ pi-sigma/pi-pi stacking/ pi anion interactions	Hydrogen bond interactions	Van der Waals forces
1	6KVP	Phytol	5280435	-6.4	LEU-272, ALA-275, ALA-279, PHE-294	-	VAL-207, LEU-270, SER-271, GLN-276, THR-296 and ILE-298
		1,2-benzene dicarboxylic acid	1017	-6.6	LEU-200, LEU-209	LEU-200, LEU-209, ASP-199, VAL-203 and GLY-205	ASN-208, GLY-295, THR-296, VAL-297 and ASN-263
		Dioctyl phthalate	8346	-7.2	MET-226, VAL-297, VAL-203, ILE-228, LEU-200, LEU-261, VAL-307, ILE-311 and ASP-199	THR-309 and ASN-263	ILE-197, THR-265, GLN-192, GLY-196, LEU-209, MET-262 and VAL-310
2	4FS3	Phytol	5280435	-6.2	ILE-20, ALA-95, TYR-147, 157 and VAL-201	LEU-102	GLY-13, SER-19, ALA-21, SER-93, MET-99, ARG-103, ASN-156, LYS-164, PRO-192, ILE-193, PHE-204, ILE-207, THR-195 and SER-197
		1,2-benzene dicarboxylic acid	1017	-6.7	ILE-94 and VAL-67	THR-38, GLY-13 and ARG-40	SER-44, TYR-39, MET-12, ILE-65, ASP-66 and ILE-120
		Dioctyl phthalate	8346	-7.6	TYR-147, VAL-154, VAL-201, TYR-157, ILE-207, PHE-204, ILE-20, ALA-95, ALA-190 and LEU-102	SER-197	GLY-13, ALA-15, SER-19, SER-93, GLN-155, ASN-156, PRO-192, GLY-191, ILE-193, THR-145, THR-146, MET-160, LYS-164, THR-195 and LEU-196

4. CONCLUSION

AMR has emerged as a global issue mainly due to unrestricted use of antibiotics, and has raised concerns on the drug effectiveness. Antimicrobial resistant nosocomial pathogens often evade antibiotic treatments; therefore, identification of novel phytochemicals can help to combat diseases where antibiotics have become ineffective. Plant derived compounds are being looked upon as unrealized resource of alternative antimicrobial drugs due to their structural complexity and functional diversity that contribute to their efficacy. Metabolite profiling of medicinal plants leads to discovery of novel drugs that could be safer and more efficient against microbial pathogens. The present study revealed the antimicrobial potential of hexane fractions of ethanolic extracts of leaf, stem and flower of *Nyctanthes arbor-tristis* against *E. coli, B. subtilis, P. fluorescens, A. hydrophila, E. faecalis* and *K. pneumonia*. A comprehensive phytochemical profiling of hexane fractions of ethanolic extracts of leaf, stem and flower of *Nyctanthes arbor-tristis* through GC-MS revealed a wide array of compounds, which established the basis of its immense therapeutic value. The GC-MS profiling led to interesting findings that included prominent presence of potential antimicrobial compounds phytol and 1,2-benzenedicarboxylic acid in the leaf and dioctyl phthalate in stem and flower. Molecular docking studies have elucidated the virtual interaction of the bioactive phytochemical ligands with the target bacterial proteins and was fairly indicative of the mechanism of antimicrobial activity of the samples. These compounds can be further isolated from complex fractions and examined for their bioactivity for future use as drug lead compounds. Standardization of procedures for extraction, bio-guided fractionation and characterization of phytochemicals would enable to isolate the accurate drug candidates. Deeper investigations into the molecular interactions leading to deciphering the mechanism of action as antimicrobial agent would guide to prepare the compounds as prospective drugs.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Prerna Sharma: Literature survey, preparation of first draft of manuscript, citation and referencing. **Abhilasha Shourie:** Design of experiments, preparation, finalization and communication of the manuscript to the journal.**Prerna Sharma** and **Abhilasha Shourie:** Experimental part involving phytochemical extraction, GC-MS antimicrobial testing, molecular docking, result interpretation and analysis.

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