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2. Research notes: These include articles such as preliminary notes on a study or manuscripts new records on secondary metabolites.

3. Reviews: Reviews of recent developments, improvements, discoveries, and ideas in various fields of plant and allied organisms (algae, fungi, and lichens) will be requested by the editor or advisory board.

4. Letters to the editor: These include opinions, comments relating to the publishing policy of the International Journal of Secondary Metabolite, news, and suggestions. Letters are not to exceed one journal page.

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Review Article

The therapeutic effects and antioxidant properties of epigallocatechin-3 gallate: A new review

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Abstract: The aim of this review is to investigate the possible protective and preventive effects of epigallocatechin-3 gallate (EGCG) in terms of human health including hepatoprotective, neuroprotective, cardioprotective, nephrotoxicity and anticancer effects. Green tea is one of the most consumed beverages in many countries, especially in Turkey and it plays protective roles in the treatment of various diseases via the polyphenol it contains. It is known that EGCG, which is the main bioactive polyphenol in green tea, has versatile bioactivities. It has been determined that EGCG has the highest free radical scavenging ability among common phenolic compounds. It regulates gene expression and molecular signaling pathways by inhibiting oxidative stress and inflammation. In addition, it prevents cell death by suppressing cytokine production and neutrophil migration in inflammatory diseases. With the studies conducted in the last decade, it has been determined that EGCG has anticancer, antioxidative, antiinflammatory, antidiabetic, antitumor, antihypertensive and neuroprotective activities. Especially, its anticancer effect has been found to have chemopreventive and chemotherapeutic activities in various cancer types such as colon, lung and breast cancer with *in vivo* and *in vitro* studies conducted to investigate molecular targets. This review was written to examine the possible protective and preventive effects of EGCG in terms of human health, including its hepatoprotective, neuroprotective, cardioprotective, nephrotoxicity and anticancer effects.

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1. INTRODUCTION

1.1. General Properties of Epigallocatechin-3 Gallate

Antioxidants and polyphenols, which have a distinctive feature in terms of human health and which are found in many plants, prevent damage to cells by reacting with free radicals (Gumuscu, 2019). Polyphenols, a group of plant metabolites that are abundant in plants and that have strong antioxidant properties, provide protection against various chronic diseases caused by oxidative stress. The consumption of dietary polyphenols by humans is of great interest due to their prevention effects of degenerative diseases and possible health benefits (Ganesan & Xu, 2017).

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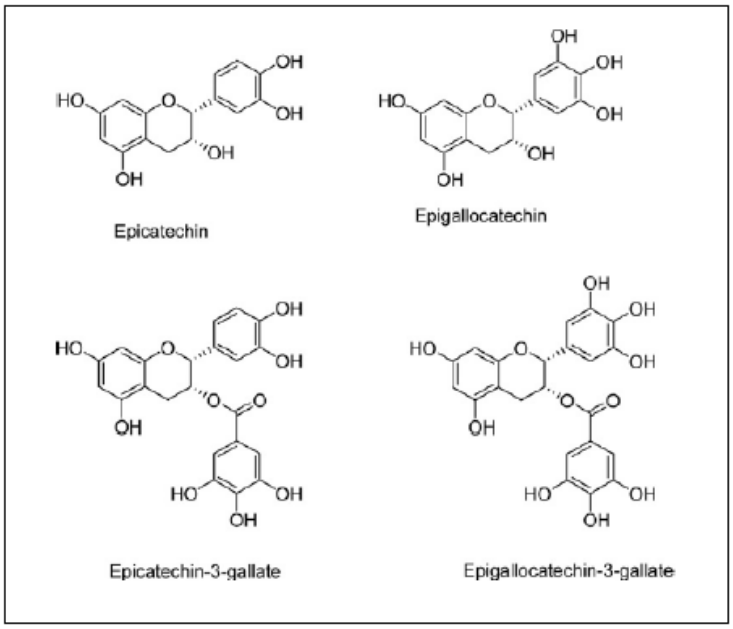
Plant-based products such as fruit, tea and coffee are very rich in polyphenol and antioxidant content (Figure 1). Tea is one of the most popular beverages around the world, both hot and cold. Different tea products such as black tea, green tea, and white tea are produced from the leaves of the *Camellia sinensis* plant with different methods applied during harvesting and processing. Since green tea is produced without oxidation, it is a rich source of unoxidized catechin (Elmas & Gezer, 2019).

Figure 1. Green tea plant (*Camellia sinensis* L.) (Guzeldir, 2015).

There are 80% polyphenols and flavonoids in the structure of green tea. Polyphenols are cyclic organic compounds bearing hydroxyl or carboxyl groups in their structures (Figure 2). Thus, they easily capture and neutralize free radicals (Sarıca, 2014; Legeay *et al.,* 2015). The phenolic compound is found in the raw extracts of green tea which consists of catechin and flavonol glycosides. Epigallocatechin-3-gallate (EGCG) is the most abundant catechin which is the main polyphenol component of green tea, nearly 50-80% of total green tea catechins. Green tea catechins have two benzene rings known as A and B rings. EGCG has two isomers in trans configuration. In addition, it has a higher capacity than vitamins C and E in terms of antioxidant capacity (Nikoo *et al.,* 2018). Green tea leaves contain carbohydrates such as cellulose, glucose, sucrose, fructose, pectins, as well as proteins, minerals, small amounts of lipids, sterols, vitamins, pigments, and volatile compounds (Figure 2) (Bilcanoglu, 2019).

Figure 2. Molecular structure of major green tea polyphenols (Guzeldir, 2015).



Recently some studies determined that EGCG, the most abundant polyphenolic catechin in green tea, has a strong antioxidant, antiinflammatory, antidiabetic, antitumor properties as well as anticarcinogenic properties. EGCG, the most active component of catechins in tea, has a very important role in ensuring DNA stability and in healthy life (Gumuscu, 2019).

In recently conducted *in vitro* and *in vivo* studies, EGCG suppresses cytokine production, endothelial activation and neutrophil migration in inflammatory disease models. In addition, it has a protective effect against ischemia-reperfusion damage in various organs, has strong antioxidant properties, and provides protection against many oxidative injuries (Chen *et al.,* 2016; Kasper *et al.,* 2016). In addition to its antioxidant, cholesterol-lowering, hypoglycemic and antihypertensive activity, it is also known as an important catechin with neuroprotective effect (Yamamoto *et al.,* 2017). It has been suggested that EGCG is a potential candidate for treatment of aging memory loss. As a neuroprotective agent, it is able to modulate various intracellular signaling pathways to alter the expression of genes involved in the regulation of cell survival and apoptosis, thereby maintaining cellular homeostasis. It is known that EGCG has some advantages in the treatment of neurodegenerative disorders and other diseases, as well as regulating cellular signal transmission pathways in cases such as antioxidation, antiapoptotic and metal chelation (Srividhya *et al.,* 2012; Zhao *et al.,* 2017). EGCG has a protective effect in normal cells against mortality and cell death due to the γ-radiation. In addition, it has been shown that it has antiproliferative and chemopreventive effects against various types of cancer such as colon cancer, lung cancer, and breast cancer (Ko *et al.,* 2013; Zhao *et al.,* 2017).

2. THE EFFECTS OF EPIGALLOCATECHIN-3 GALLATE ON HEALTH

2.1. Cardioprotective Effects of Epigallocatechin-3 Gallate

Cardiovascular diseases occur due to the abnormal function of the heart and blood vessels, being one of the leading causes of deaths worldwide. In recent years, herbal medicines are used in the treatment of cardiovascular diseases due to their low side effects rather than pharmaceutical drugs. In many herbal medicines, it has been determined that green tea has a high therapeutic potential against cardiovascular diseases. EGCG, a bioactive polyphenol in green tea, has a lot of biological and pharmacological activities in the treatment of many diseases other than its cardiovascular protection (Liu *et al.,* 2014; Luo *et al.,* 2017).

Eng *et al.* (2018) reported that EGCG inhibits the activation of the NF-κB signaling pathway. Oyama *et al.* (2017) stated that EGCG has an effective role in protecting the cardiovascular system. Othman *et al.* (2015) investigated the potential protective effect of EGCG against cardiovascular diseases caused by type-2 diabetes and stated that glutathione levels and catalase activities increased in the EGCG treatment groups. Saeed *et al.* (2015) investigated the protective role of EGCG against doxorubicin (DOX) induced cardiotoxicity in rats and reported that EGCG provides protection against DOX-induced cardiomyopathy. In addition, Al Hroob *et al*. (2019) stated that EGCG has a therapeutic effect against the processes involved in the pathogenesis and progression of diabetic cardiomypathy and found that EGCG therapy is a promising drug to reduce diabetes-related morbidity and mortality. Meng *et al.* (2020) concluded that EGCG induces autophagy by targeting the autophagy-related PI3K-AKT-mTOR pathway and that EGCG prevents and treats oxidative stress-induced cardiovascular diseases. Zeng *et al.* (2021) investigated the protective effects and molecular mechanism of EGCG against myocardial ischemia/reperfusion injury (I/RI) and noted that EGCG protects against myocardial I/RI.

2.2. Effects of Epigallocatechin-3 on Nephrotoxicity

The kidneys are responsible for keeping the osmotic pressure of the blood stable by maintaining fluid-electrolyte balance, cleansing the body from metabolic wastes such as urea, creatine, uric acid and contributes to the regulation of acid-base balance. In addition, the kidneys are a

homeostasis center, the production site of some vital hormones such as renin and erythropoietin, thus they balance the uptake, production, excretion and retention of many organic and inorganic compounds (Yıldıran & Gencer, 2018; Koken, 2018).

This balance ensures that the kidneys retain water and water-soluble substances and the content of body fluids by excreting water according to body needs (Yıldıran & Gencer, 2018; Koken, 2018). Kidney diseases are an important public health problem in the world and in our country. Approximately 10% adults of all over the World are in various stages of kidney disease, while this has been estimated to be approximately 15.7% in Turkey. Increasing use of medicinal plants offers new solution possibilities in the treatment of kidney diseases, which are the target of many cytotoxic substances. In recent studies, it has been determined that EGCG protects kidney tissue against oxidative stress and nephrotoxicity (Cellat & Kılıcalp, 2010; Yalcın *et al.,* 2017). Thanks to its antioxidant effect, EGCG has been determined to protect kidney tissue against oxidative stress and necrosis, cleanses free radicals, and treats kidney tissue damage. In addition, it has been determined that it prevents the loss of function of kidney (Cellat & Kılıcalp, 2010; Yalcın *et al.,* 2017).

Zhang and Zhang (2018) found that EGCG significantly reduces the nephrotoxic effect in kidney tissue by providing antioxidant defense. Zhu *et al.* (2018) demonstrated that EGCG has potential value in the treatment of significant hypouremic and hyperuricemia. Peng *et al.* (2011) investigated the effects of EGCG on immune-mediated glomerulonephritis (GN) and concluded that EGCG significantly reduces renal impairment. They also concluded that EGCG has a therapeutic effect for the treatment of immune-mediated GN and other immune-mediated diseases.

2.3. Neuroprotective Effects of Epigallocatechin-3 Gallate

Neurodegenerative diseases occur as a result of advanced degeneration of nerve cells and cause socio-economic negativity on the society. Neurodegenerative diseases such as Alzheimer's and Parkinson's are caused by the accumulation of modified proteins that further trigger inflammation, oxidative stress, and modulation of signaling pathways. EGCG, one of the green tea polyphenols, has a wide spectrum of biological and pharmacological activity and provides very strong protection in the treatment of neurodegenerative diseases. Thus, EGCG prevents neuronal damage by reducing brain inflammation in *in vivo* and *in vitro* studies (Singh *et al.,* 2015).

Zhao *et al.* (2017) revealed that EGCG has neuroprotective and neuro-restorative effects against various brain injuries, including some neurodegenerative diseases. They noted that EGCG therapy has a potential therapeutic drug capacity to prevent neurodegenerative diseases, cerebral trauma, and other related pathogenesis. They also concluded that EGCG treatment could reduce cerebral disorders due to psychological stress in rats.

Khalatbary and Khademi (2020) found that EGCG has neuroprotective effects and is responsible for neuroprotection in various neurodegenerative and neural injury models. Pervin *et al.* (2018) stated that EGCG provides effective protection by preventing abnormal accumulation of fibrous proteins such as Aβ and a-synuclein, inflammation, and neuronal cell dysfunction in the cerebral cortex in Alzheimer's patients.

2.4. Hepatoprotective Effects of Epigallocatechin-3 Gallate

The liver is an important metabolic organ in the body and is responsible for secreting and processing various nutrients into proteins (Zhou *et al.,* 2015). Recently, it has been determined that EGCG treatment reduces acid homeostasis, lipid metabolism, and fatty liver (Huang *et al.,* 2018). Liao *et al.* (2019) stated that EGCG inhibits the proliferation of liver cancer cells as well as the formation and development of liver cancer.

Naito *et al.* (2020) investigated the effect of EGCG treatment on intestinal microbiota, serum acid, and gene expression in the liver in mice fed a high-fat diet and they found that EGCG significantly inhibits excessive fat accumulation in the liver. Tipoe *et al.* (2010) stated that EGCG inhibits the NF-κB signalling pathway due to its antioxidant effects against carbon tetrachloride (CCl_4) induced liver damage in rats. Rishi *et al.* (2017) found that EGCG treatment protects against liver damage.

2.5. Effects of Epigallocatechin-3 Gallate on Visual Impairment

Visual disturbances develop due to age or various factors that affect human life negatively. It makes life difficult not only physically but also spiritually. It is known that EGCG has positive effects in the treatment of visual disorders. Recently conducted studies indicate that EGCG treatment is highly effective in the treatment of eye tissue damage (Lee *et al.,* 2011). Shen *et al.* (2015) stated that EGCG provides protection against the degeneration of retinal ganglion cells in an animal model of glaucoma.

Qi *et al.* (2017) found that EGCG treatment provides highly effective protection against light-induced photoreceptor degeneration in the Balb/c mouse retina. Kumar *et al*. (2017) stated that EGCG treatment has a strong drug potential in preventing the onset of cataracts and in the treatment of cataracts.

Falsini *et al.* (2009) found that EGCG treatment could positively affect the inner retina function of the eyes against glaucoma damage. Lee *et al.* (2011) concluded that EGCG treatment inhibits the inflammatory cytokine expression in the rat cornea dry eye model.

2.6. Anticancer and Antitumor Effect of Epigallocatechin-3 Gallate

EGCG, an ingredient derived from green tea, has many effects on human pathological and physiological processes (Chu *et al.,* 2017). It is known that it plays an important role in the regulation of gene expression and transcription (Chu *et al.,* 2017). These effects come from its polyphenolic compounds (catechins) which are found in green tea. EGCG induces apoptosis in various cancer cells by inhibiting cell proliferation. In addition, it stops the growth of cancer cells by selecting cancer cells without affecting normal cells (Safwat *et al.,* 2020).

Shankar *et al.* (2013) stated that EGCG treatment inhibits tumor growth by decreasing the phosphorylation of genes such as Erk/Akt and suppresses apoptosis by activating caspase-3 expression. Abd El-Rahman *et al.* (2017) investigated the effect of EGCG on 7,12 dimethylbenz[a]anthracene-induced breast tumor metastasis and anticancer in rats. They stated that EGCG decreased significantly the tumor size and tumor amount. Lecumberri *et al.* (2013) stated that when combined with traditional cancer therapies, EGCG can provide an additional synergistic effect in improving cancer therapy side effects along with antiinflammatory and antioxidant activities. Zhu *et al.* (2017) stated that EGCG effectively reduces lung cancer stem cell activity by inhibiting tumor formation and inducing apoptosis. Shirakami *et al.* (2009) investigated the effect of EGCG on hepatocellular carcinoma tumor and stated that EGCG causes a significant decrease in Erk/Akt protein levels.

Cerezo-Guisado *et al.* (2015) found that EGCG induces cell death in colon cancer cell line (HT-29 cells) and it has cytotoxic effects and has a cancer preventive role. Yang *et al.* (2019) stated that EGCG induces apoptosis by inhibiting proliferation in odontogenic keratocyst keratinocytes by suppressing the WNT/JNK signaling pathway. Jankun *et al.* (2014) stated that EGCG had better efficacy to prevent tumor cell growth than mitomycin C (MMC) when they compared EGCG and MMC in rats with superficial bladder cancer. Tepedelen *et al.* (2021) investigated the role of EGCG in the transcriptional regulation of genes related to inflammation and migration in benign prostatic hyperplasia cells. They stated that EGCG treatment was effective in the treatment of premalignant lesions by reducing NF-κB and FAK protein expression levels.

Luo *et al.* (2020) determined that EGCG treatment increased p53 protein expression and exhibited antitumor activity by decreasing NF-κB and Bcl-2 protein expression levels against doxorubicin (DOX)-induced bladder cancer.

2.7. Antioxidant Effect of Epigallocatechin-3 Gallate

EGCG is classified as an antioxidant according to its chemical structure it acts as scavengers of free radicals with electrons in phenol rings in the EGCG structure, has redox properties like other tea catechins, prevents the formation of reactive oxygen species, and reduces the damage caused by oxidative stress (Chu *et al.,* 2017; Bimonte *et al.,* 2019).

Fouad *et al.* (2017) stated that EGCG can provide testicular protection with its antioxidant, antiinflammatory and antiapoptotic effects in rats induced with cisplatin. Abib *et al.* (2011) stated that EGCG protects rat brain mitochondria against cadmium-induced damage and found that EGCG completely prevents mitochondrial lipid peroxidation due to its antioxidant and chelating effects. Hassan *et al.* (2019) investigated the therapeutic and protective effects of EGCG on infertility due to lead toxicity in male rats and found that EGCG lowers tissue malondialdehyde levels, protects antioxidative enzyme levels, and provides significant protection against testicular toxicity caused by the lead. Tseng *et al.* (2015) stated that EGCG, through its powerful antioxidative activities, has a protective effect in the treatment of memory impairment caused by reserpine. He *et al.* (2017) stated that treatment of PC12 cells with EGCG reduced oxidative stress and alleviated apoptosis, increased superoxide dismutase activity, glutathione levels, but decreased oxidative stress by inhibiting the formation of reactive oxygen species and lipid peroxidation.

Lee *et al.* (2003) found that EGCG, trolox, lipoic acid, and melatonin reduced lipid peroxidation caused by H_2O_2 or iron ion in a concentration-dependent manner. They stated that EGCG is the most powerful antioxidant in inhibiting lipid peroxidation in gerbil brain homogenates under *in vitro* conditions. Zong *et al.* (2021) stated that EGCG is a protective agent against aminoglycoside-induced ototoxicity. They also found that EGCG could significantly reduce the number of apoptotic cells induced by amikacin and gentamicin. Wei *et al.* (2021) determined that EGCG reduces oxidative stress by regulating the activities of antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) and that EGCG could be an effective bioactive compound to reduce cyclophosphamide-induced gastrointestinal toxicity.

2.8. The Effect of Epigallocatechin-3 Gallate on Obesity

Obesity is one of the main health problems of the developed and developing countries today. Obesity is the result of a balance triggered by the consumption of excessive calorie foods and reduced physical activity. It consists of adipocytes, pre-adipocytes, immune cells and endothelial cells and is responsible for the long-term storage of lipids. Accumulating excessive amounts in the body causes oxidative stress and inflammation in the lipid cell. As a result of pharmacokinetic studies, it is known that EGCG increases the burning of excess fat accumulated in adipose tissue and increases the metabolic rate (Legeay *et al.,* 2015).

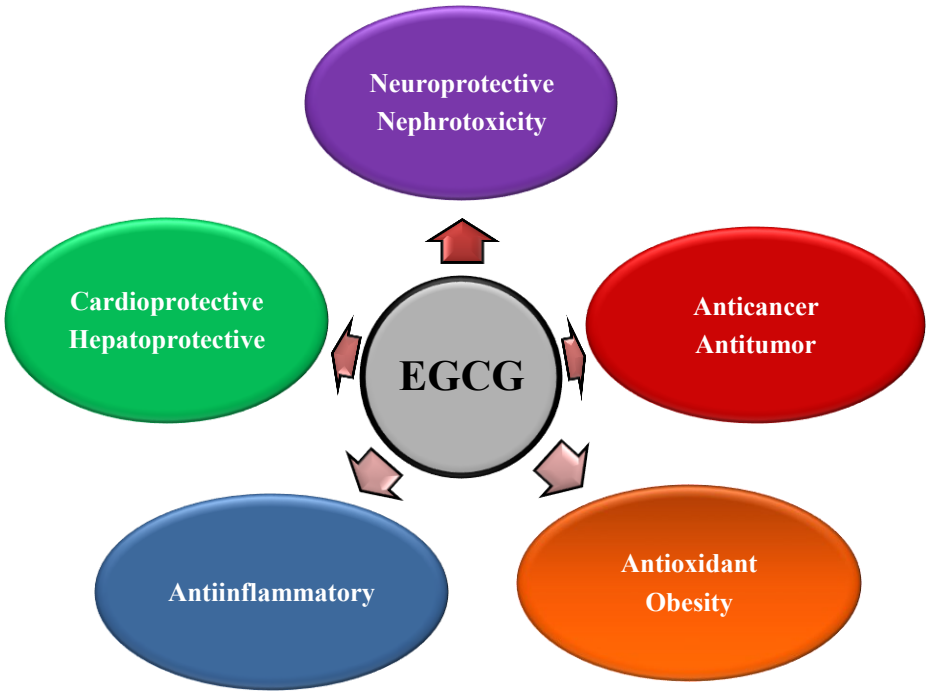
Fiorini *et al.* (2005) investigated the effects of EGCG on obesity in leptin-deficient mice and found that 85 mg/kg EGCG treatment for 5 days accelerates the reduction in body weight in obese mice. Snoussi *et al.* (2014) reported that daily administration of EGCG led to a reduction in body weight within one week in male rats fed a fatty diet (22% fat, 43% carbohydrate and 21% protein). Legeay *et al.* (2015) reported that EGCG can prevent obesity through a modulation involving different organs such as adipose tissue or the liver and also noted that EGCG consumption inhibits pancreatic lipase *in vitro* and suppresses postprandial serum triglycerides in a dose-dependent manner.

3. CONCLUSION

Herbal products have been widely used for the treatment of metabolic diseases all over the world since ancient times. Green tea, one of the plants rich in polyphenols, has powerful therapeutic activities thanks to its epigallocatechin-3 gallate (EGCG) content.

According to the results obtained from epidemiological studies, it has been determined that EGCG positively affects a number of signalling and metabolic pathways, leading to the improvement of various symptoms (Figure 3). Accordingly, EGCG has proven to have an extremely effective drug capacity in terms of human health.

Figure 3. Biological activities of epigallocatechin-3 gallate.



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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

Seda Beyaz: Writing, reading and editing the original draft of the article as well as researching its content. **Ozlem Gok:** Investigation, resources and writing original draft. **Abdullah Aslan:** Writing, reading and editing of article; conducting the study.

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Research Article

Antioxidant Activities and Chemical Composition of Essential Oil of Rhizomes from *Zingiber officinale* R. (Ginger) and *Curcuma longa* L.(Turmeric)

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Abstract: This study aimed to determine the essential oil volatile components of ginger and turmeric rhizomes, as well as to determine the total antioxidant capacity of essential oil samples according to the CUPric Reducing Antioxidant Capacity (CUPRAC), ferric reducing antioxidant potential (FRAP) method and free radical scavenging activities of oil samples and standards such as BHA, BHT, and Trolox were determined using a DPPH method. Essential oil analysis of volatile components was also performed on a Shimadzu GCMS-QP2010 SE (Japan) model with Support Rx-5Sil MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). Antioxidant capacities of essential oils were evaluated according to the CUPRAC method in millimole Trolox/gram -oil equivalent. GC-MS analysis of ginger showed the presence of 5 major peaks identified as Curcumene (13.46%), Zingiberene (33.92%), α-Farnesene (8.07%), β-Bisabolene (6.39%), and β-Sesquiphellandrene (15.92 %), respectively. GC-MS analysis of Turmeric showed the presence of 3 major peaks identified as Ar-Turmerone (29.24%), α-Turmerone (22.8 %), and β-Turmerone (18.84%). CUPRAC values of calculated antioxidant capacities of essential oil samples were determined as 1.97 ± 0.102 mmolTR/g-oil for *Zingiber officinale* R. and 3.40 ± 0.071 mmol TR/g-oil for *Curcuma longa* L. The scavenging effect of turmeric, ginger and standards on the DPPH radical decreased in the order of Trolox>BHA>BHT>Turmeric>Ginger which were 95.25 ± 0.05%, 62.57 ± 0.34%, 61.6 ± 0.3%, 51.45 ± 0.59%, and 50.26 ± 0.09%, at the concentration of 150µg/mL, respectively. Additionally, it revealed that essential oils of turmeric and ginger exhibited effective ferric reducing power.

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1. INTRODUCTION

Essential oils are volatile, strong-smelling, and oily mixtures obtained from plants by hydrodistillation of water or water vapor, liquid at room temperature, but can sometimes freeze. They are called "essential oil" or "etheric oil", because they can evaporate even under room temperature and "essence" because they are fragrant. Essential oils obtained from spices have been used since ancient times for their perfume, medicinal and preservative properties, and

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adding aroma and flavor to food (Bilia *et al.,* 2014). Essential oils are generally complex mixtures of volatile organic compounds produced as secondary metabolites in plants. Essential oils consist of components belonging to the terpene and phenylpropanoid groups, such as monoterpenes and sesquiterpenes, where the main compounds usually determine the biological properties of the essential oil (de Cássia Da Silveira e Sá *et al.,* 2015). Terpenes are constructed from combinations of several 5-carbon-based (C5) units called isoprenes, forming structurally and functionally diverse classes (Bilia *et al.,* 2014). Sesquiterpenes, consisting of a combination of three isoprene units (C15), are a subclass of terpenes that have been described to exhibit a broad spectrum of biological and pharmaceutical activities (Moujir *et al*., 2020).

Antioxidant components can sweep free radicals and prolong shelf life by delaying the lipid peroxidation process, which causes food and pharmaceutical products to deteriorate (Halliwell, 1996). In general, antioxidants act by chain-breaking reactions, reducing the concentration of reactive oxygen species, scavenging initiating radicals, and chelating transition metal catalysts (Eroğlu *et al.,* 2015). An inquiry of normally happening antioxidant ingredients from plant sources may prompt the advancement of novel medicines, which may diminish the danger of long-term infections brought about by free radicals (Abuja & Albertini, 2001). Many methods based on free radical scavenging have been developed to determine antioxidant capacity in recent years. The CUPric Reducing Antioxidant Capacity (CUPRAC) method is a simple and versatile antioxidant capacity method for applying many different components, including nutritional components, synthetic antioxidants, and vitamins C and E (Özyürek *et al.,* 2011).

Turmeric (*Curcuma longa* L.) is a perennial plant that belongs to the Zingiberaceae family and is widely cultivated in Asian countries. *Curcuma longa* L. rhizomes are used in many fields such as textile, medicine, cosmetics, and food (Singh *et al.,* 2003). The rhizomes of this plant are the most useful and are used for culinary and traditional medicinal purposes (Bagchi, 2012). Turmeric rhizomes are widely used as a spice in Indian and Mediterranean cuisine. It is frequently used for many therapeutic purposes in alternative medicine. Turmeric is also used in medicines to treat cancer, dermatitis, AIDS, and high cholesterol (Ammon, & Wahl, 1991; Kuttan *et al.,* 1985). Curcumin is the most important bioactive component of turmeric, which is also used as a spice (Martín-Cordero *et al*., 2003). Investigations of turmeric have uncovered various pharmacological properties (Huei-Chen *et al.,* 1992; Wichitnithad *et al.*, 2009). However, Ginger, whose Latin name is *Zingiber officinale*, is a plant of the Zingiberaceae family, growing up to one meter in length, with long leaves and yellow-red flowers. The antioxidant, antiseptic and carminative properties of many different bioactive components of ginger have made its use popular (Mushtaq *et al*., 2019). Also, the essential oil from ginger has been found to have antibacterial, antiviral, and antifungal properties (Koch *et al*., 2008; Singh *et al*., 2005). Our previous study has already reported that the phytochemical profile of ethanolic extraction from both rhizomes is very rich (Erdoğan & Erbaş, 2021).

This study aimed to determine the essential oil volatile components of ginger and turmeric rhizomes and determine the total antioxidant capacity of essential oil samples according to the CUPRAC, FRAP, and DPPH methods.

2. MATERIAL and METHODS

2.1. Chemicals

Copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox, Neocuproine (Nc-$C_{14}H_{12}N_2$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Absolute ethanol (EtOH) was purchased from ISOLAB Laborgeräte GmbH (Eschau, GERMANY**).** Potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), Iron(III)

chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), trichloroacetic acid (TCA), and Ammonium acetate (NH_4Ac) were purchased from Merck (Darmstadt, Germany).

2.1. Preparation of Solutions

All CUPRAC reagents were prepared by dissolving in a small amount of distilled water and then diluting with ethanol. The copper (II) chloride solution was prepared by weighing 0.085 g of $CuCl_2.2H_2O$ to be 1.0×10^{-2} M, dissolving it in a small amount of distilled water and diluting it to 50 mL with ethanol. Ammonium acetate buffer is prepared by weighing 7.71 g at 1 M (pH=7), dissolving it in a small amount of distilled water, and diluting it to 100 mL with ethanol. Neocuproin solution was prepared by weighing 0.78 g as 7.5×10^{-3} M and diluting to 50 mL with ethanol.

The FRAP reagents were prepared as follows: To prepare 0.2 M phosphate buffer at pH 6.6, 7.80 g of $NaH_2PO_4 \cdot 2H_2O$ was dissolved in water and diluted to 250 mL with H_2O such that its final concn. would be 0.2 M; 8.90 g of $Na_2HPO_4 \cdot 2H_2O$ was dissolved in water and diluted to 250 mL such that its final concn. would be 0.2 M. To prepare 0.2 M phosphate pH 6.6 buffer, 62.5 mL of $NaH_2PO_4 \cdot 2H_2O$ solution was mixed with 37.5 mL of $Na_2HP0_4 \cdot 2H_2O$ and diluted to a total of 200 mL with H_2O (Stoll & Blanchard, 2009). Potassium ferricyanide solution (1%, w/v) was prepared daily by dissolving 1 g $K_3Fe(CN)_6$ in 1 mL of 1 M HCl and some water and diluting to 100 mL with water. Ferric chloride solution (0.1%, w/v) was prepared daily by dissolving 0.1 g of $FeCl_3 \cdot 6H_2O$ in 1 mL of 1 M HCl and some water and diluting to 100 mL with water. Trichloroacetic acid (TCA) solution (10%, w/v) was prepared by dissolving 10 g of TCA in water and diluting it to 100 mL with H_2O (Berker *et al.,* 2007).

2.3. Plant Material

The turmeric and ginger were obtained from the Isparta University of Applied Sciences Faculty of Agriculture. Plant specimens were also identified by Prof. Hasan Baydar and deposited at the herbarium of Faculty of Agriculture, Isparta University of Applied Sciences, with voucher specimen numbers: TP32-2020 and GP32-2020, respectively.

2.4. Essential Oil Isolation

The isolation procedure of the essential oil is as follows; 100 g of both types of rhizome preparations were subjected to hydrodistillation, separately, in a Clevenger apparatus for 4 h. From 100 grams of turmeric and ginger powder samples, 3.15 and 1.65 mL of pure essential oil were obtained, respectively. The essential oils obtained were kept at +4 ºC until used in the analysis.

2.5. Essential Oil Components Analysis with GC-MS

Essential oil analysis of volatile components was performed on a Shimadzu GCMS-QP2010 SE (Japan) model with Support Rx-5Sil MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). GC analyses were performed under the following conditions (Erdoğan *et al*., 2020). The carrier gas (helium) flow rate was 1 ml/min. The split ratio was 1:10. After 1 min at 60 ºC, the temperature program reached 250 ºC with an increase of 4 ºC per min and was kept at 250 ºC for 15 min. The mass spectra were taken at 70 eV. 970 µL hexane was added over 30 µL of pure essential oil. 1 µL was injected from the capped vial. The identification of the separated compounds was made based on a comparison of the mass spectra obtained with NIST27 and NIST147 from the US National Institute of Technology and Standards (NIST) mass spectra libraries.

2.6. CUPRAC Assay of Total Antioxidant Capacity

Total antioxidant analysis of oil samples was done by modifying the CUPRAC method developed by Çelik *et al.* (2019). The method has been modified to be applied to oil samples.

Therefore, CUPRAC reagents were prepared fresh daily in ethanol medium. Briefly, to a test tube were added 1 mL each of ethanolic Cu(II), Nc, and NH_4Ac buffer solutions. Then a 0.5 mL oil sample diluted with acetone at a specific ratio (1:100, v/v) and 0.6 mL of EtOH were added. The tubes were closed, and after 30 min, the absorbance at 450 nm (A450) was recorded against a reagent blank. According to the equation below, the total antioxidant capacity of the oil samples was calculated as mmol/g Trolox equivalent. If the absorbance of the sample was greater than 2 when the CUPRAC method is applied, the extract should be diluted at an appropriate ratio, and the measured absorbance should be in the range of 0.2<A<1.5 in order to prevent deviations from the Lambert-Beer law. The assays were carried out in triplicate, and the results were expressed as (mean values ± standard deviations).

Reagent blank solution: 1 mL Cu(II) + 1 mL Nc + 1 mL NH_4Ac + 1.1 mL ethanol

Sample solution: 1 mL Cu(II) + 1 mL Nc + 1 mL NH_4Ac + X mL sample + (1.1-X) mL ethanol

$$TAC(mmolTR\ /\ g-oil) = \frac{A}{\varepsilon} x \frac{V_t}{V_{\ddot{o}}} x\ S.f x \frac{Ve}{m}$$

Where;
A: Sample absorbance measured at 450 nm
Ɛ: Molar absorption coefficient of TR compound in the CUPRAC method (16700 L $mol^{-1}.cm^{-1}$) (Çelik *et al.,* 2010)
Vt: Total volume of CUPRAC measuring solution (4.1 mL)
Vö: Sample volume (mL)
S.f.: Dilution factor (if no dilution will be made, this factor is taken as "1")
Ve: Volume of the prepared extract (mL)
m: The amount of sample taken in the extraction process (g)

2.7. Free Radical-Scavenging Activity on DPPH

The free-radical-scavenging capacity of oil samples was evaluated, using the DPPH• stable radical and following the methodology described by Blois (1958). The free radical scavenging capacity of pure essential oil samples was determined by considering the recommendations on using DPPH radicals in Molyneux's study (2003). Briefly, 0.1mM solution of DPPH• in ethanol was prepared, and 2 mL of this solution was added to 2 mL of oil sample solution at 150 ug/mL concentration in ethanol medium. After 30 min, the absorbance was measured at 517 nm against ethanol as a blank in a spectrophotometer (SHIMADZU UV-1280 UV-Vis Spectrophotometer).

The ability to sweep the DPPH• radical was counted up using the following equation: DPPH• scavenging effect (%) = $[(A_{Control} - A_{Sample} / A_{Control}) \times 100]$ where $A_{Control}$ was the absorbance of the control reaction (ethanol solution containing 0.1 mM DPPH•) and A_{Sample} was the absorbance in the presence of oil samples and standards (BHT, BHA, Trolox).

2.8. Ferriccyanide (Fe^{3+}) Reducing Antioxidant Power Assay

Procedure. The reducing capacity (RP) of the extracts was assessed as described by Oyaizu (1986). 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ solution (1%) were added to 1 mL of oil sample solution at different concentrations (500-1000 µg/mL) in ethanol); the mixture was incubated at 50 ˚C on a water bath for 20 min. The incubated mixture was let to cool to room temperature, and 2.5 mL of TCA (10%) was added. The solution was thoroughly mixed by vortexing for 30 s., an aliquot of 2.5 mL was withdrawn from the supernatant, and 2.5 mL water was followed by 0.5 mL of $FeCl_3 \cdot 6H_2O$ solution (0.1%) added so that the final volume was 5.5 mL. The colored solution was read at 700 nm against the blank regarding standard using UV Spectrophotometer (SHIMADZU UV-1280 UV-Vis Spectrophotometer). BHA and BHT were used as standard references.

3. RESULTS and DISCUSSION

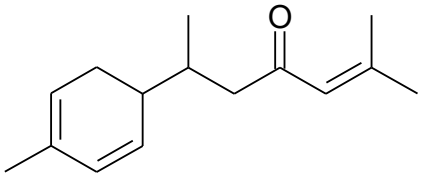
3.1. Essential Oil Efficiency

From 100 grams of turmeric and ginger powder samples, 3.15 and 1.65 mL of pure essential oil were obtained, respectively. In a study presented in the literature, essential oil isolation from turmeric was performed using a modified microwave distillation system and a rotary evaporator unit. This study determined that essential oils ranging from 1.895% to 4.973% were obtained from turmeric rhizomes (Sachin *et al.,* 2020). On the other hand, it has been reported that the essential oil obtained from ginger varies between 1% and 4% depending on the region and variety (El-Ghorab *et al.,* 2010). It was determined that the results obtained in our study were consistent with the data presented in the literature.

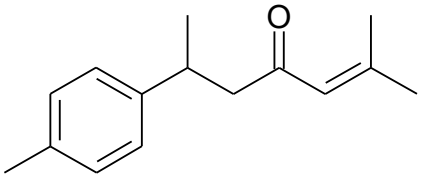
3.2. Chemical Composition of The Essential Oil of Dry Rhizomes from *Zingiber officinale* and *Curcuma longa*

Almost all (about 90%) of *Z. officinale* essential oil consists of sesquiterpenes components. Sesquiterpenes are molecules in the composition of essential oils and are responsible for the pharmacological activity of essential oils. The GC-MS analysis of *Z. officinale* rhizome (Table 1) showed the presence of 5 major peaks distinguished at 38.624, 39.663, 40.235, 40.341, and 41.31 min, which were identified as ar-Curcumene (13.46%), Zingiberene (33.92%), α-Farnesene (8.07%), β-Bisabolene (6.39%), and β-Sesquiphellandrene (15.92 %) respectively (Figure 1). It has been reported that these components have many different bioactivities such as antioxidant (Marliyana *et al*., 2019), antimicrobial (Pulido-Moran *et al.,* 2016), antiaging (Nelson *et al*., 2017), and anticancer (Naksuriya *et al.,* 2014). Simultaneously, 40 minor compounds were also identified, presented in Table 1. Other researchers also reported similar results concerning the content of major constituents (Pino *et al*., 2004; Sasidharan & Menon, 2010). On the other hand, Approximately 80% of the essential oil obtained from *C. longa* rhizomes consists of sesquiterpenes. GC-MS analysis of *C. longa* rhizome (Table 2) showed the presence of 3 major peaks distinguished at 49.548, 49.85, and 51.62 min, which were identified as Ar-Turmerone (29.24%), α-Turmerone (22.8 %), and β-Turmerone (18.84%), respectively. Simultaneously, 23 minor compounds were also identified as minor compounds were presented in Table 3. These findings were compatible with many studies in the literature (Gopalan *et al*., 2000; Zaeoung *et al*., 2005).

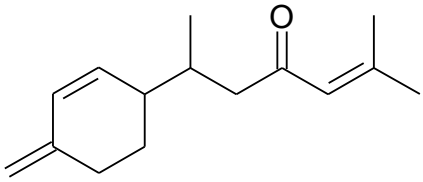
Figure 1. Major compononents of essential oil from *Zingiber officinale* R. and *Curcuma longa* L. Rhizome.



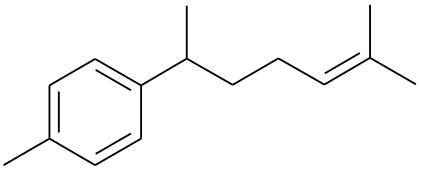
α-Turmerone



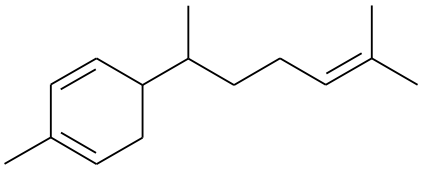
Ar-Turmerone



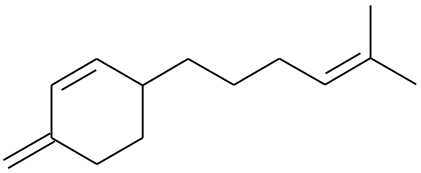
β-Turmerone



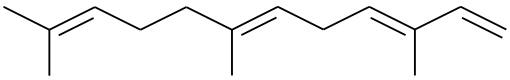
Ar-Curcumene



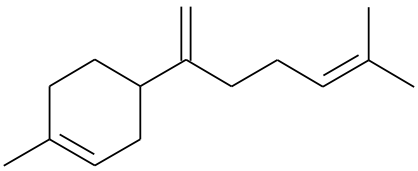
Zingiberene



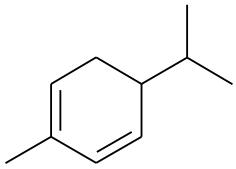
β-Sesquiphellandrene



α-Farnesene



β-Bisabolene



α-Phellandrene

Table 1. Chemical composition of *Zingiber officinale* (Ginger) essential oil.

Compound[a]	RI[b]	R.Time	% area
Camphene	950.3	7.258	0.33
β-Phellandrene	1030.0	9.485	0.83
Eucalyptol (1,8-cineole)	1031.8	10.694	0.5
Linalool	1099	14.263	0.32
β-Terpineol	1143.9	17.400	0.11
Borneol	1166.2	18.489	1.58
Terpinen-4-ol	1177.1	19.044	0.29
α-Terpineol	1189.7	19.986	0.89
Nerol	1228.9	23.698	0.11
Neral	1242.1	22.777	0.23
Linalyl acetate	1255.2	32.079	0.44
Geranial	1270.3	24.735	0.39
Isobornyl acetate	1285.9	25.767	0.22
2-Undecanone	1293.1	26.462	0.85
Citronellyl acetate	1352.4	30.219	0.18
Cyclosativene	1368.2	31.110	0.26
α-Ylangene	1369.9	38.308	1.11
α-Copaene	1376.2	31.672	0.61
β-Elemene	1390.4	32.622	0.38
7-epi-Sesquithujene	1393	33.574	0.27
α-Gurjunene	1408.6	36.264	0.11
β-Caryophyllene	1420.1	36.883	1.25
α-Bergamotene	1434.5	49.701	0.49
γ-Elemene	1436.4	35.178	0.17
α-Guaiene	1439.6	47.392	0.17
α-Patchoulene	1457.2	41.406	0.38
ε-Muurolene	1458.8	36.392	0.28
γ-Gurjunene	1472.2	47.010	0.43
ar-Curcumene	1482.2	38.624	13.46
Eudesma-4(14),11-diene	1486.1	38.790	0.47
Valencene	1491.7	41.821	0.17
α-Zingiberene	1495.3	39.663	33.92
β-Himachalene	1501.0	54.060	0.12
α-Farnesene	1504.1	40.235	8.07
β-Bisabolene	1508.4	40.341	6.39
γ-Cadinene	1513.1	38.025	0.67
Δ-Cadinene	1523.2	40.810	1.43
β-Sesquiphellandrene	1523.5	41.311	15.92
Elemol	1547.5	42.524	0.3
Germacrene B	1550.9	43.010	0.38
α-Cedrol	1600.1	50.710	1.18
γ-Eudesmol	1630.9	46.733	0.2
Murolan-3,9(11)-diene-10-peroxy	1730	42.063	0.43
Farnesol	1743.5	43.453	0.81
α-Springene	1940	51.119	0.77
Monoterpene hydrocarbons (%)			1.82
Oxygenated monoterpenes (%)			4.60
Sesquiterpene hydrocarbons (%)			86.91
Oxygenated sesquiterpenes (%)			2.92
Others (%)			1.62
Total (%)			97.87

[a]Compounds were listed in order of their elution from a Restek Rxi®-5Sil MS column using a series of the standards of C_7-C_{30} saturated n-alkanes. [b]Retention index from the literature (Adams, 2007; Babushok *et al.,* 2011)

Table 2. Chemical composition of *Curcuma longa* L.(Turmeric) essential oil.

Compound	RI	R.Time	% area
Bornylene	908	10.558	0.39
α-Pinene	936.1	6.686	0.17
β-Myrcene	989.2	8.742	0.16
α-Phellandrene	1004.1	9.499	4.2
Δ-3-Carene	1011.3	9.612	0.1
Eucalyptol	1031.8	10.698	1.76
O-Cymene	1041	10.325	2.47
γ-Terpinene	1059.7	12.004	0.17
Terpinolene	1086.9	13.455	0.29
β-Fenchyl Alcohol	1100.7	19.962	0.11
Phenethyl alcohol	1114.9	19.443	0.1
Camphor	1143.4	47.845	0.12
α-Longipinene	1352.1	45.072	0.61
2,2,4,4,7,7-Hexamethyl-2,3,3a,4,7,7a-hexahydro-1H-indene	1367	55.455	2.69
Sesquithujene <7-epi->	1393	39.383	2.6
β-Caryophyllene	1420.1	34.391	0.61
β-Farnesene <(E)-	1455.9	36.826	0.21
ar-Curcumene	1482.2	38.468	2.51
β-Bisabolene	1508.4	40.165	0.73
β-Sesquiphellandrene	1523.5	41.111	2.97
6,10-Dodecadien-1-yn-3-ol, 3,7,11-trimethyl-	1562	48.180	0.16
α-Cedrol	1600.1	42.956	0.86
α-Turmerone	1631	49.859	22.8
β-Turmerone	1647	51.622	18.84
Ar-Turmerone	1664	49.548	29.24
cis α-Santalol	1683	45.989	0.6
(Z)-valerenyl acetate	1804	47.693	0.96
Monoterpene hydrocarbons (%)			5.09
Oxygenated monoterpenes (%)			1.87
Sesquiterpene hydrocarbons (%)			11.2
Oxygenated sesquiterpenes (%)			72.11
Others (%)			5.81
Total (%)			96.2

[a]Compounds were listed in order of their elution from a Restek Rxi®-5Sil MS column using a series of the standards of C_7-C_{30} saturated n-alkanes. [b]Retention index from the literature (Adams, 2007; Babushok *et al.,* 2011)

3.3. Antioxidant Capacity of *Zingiber officinale* and *Curcuma longa* Essential Oil

The antioxidant capacity of essential oils is most likely due to the interaction between their main components. The antioxidant capacity of essential oil samples was evaluated according to the CUPRAC, FRAP, and DPPH methods. CUPRAC reagent has more stable and accessible advantages over other chromogenic reagents (e.g., ABTS, DPPH) (Apak *et al.,* 2008). CUPRAC values of calculated antioxidant capacities of essential oil samples were determined as 1.97± 0.102 mmolTR/g-oil for *Zingiber officinale* and 3.40 ± 0.071 mmol TR/g-oil for *Curcuma longa*. When the data were analyzed, the antioxidant capacity of turmeric essential oil was greater than ginger.

In the FRAP method, the reducing capacity of oil samples was accomplished using Fe^{3+} to Fe^{2+} reduction assay. In this analysis, the yellow color of the frap test solution changed to shades of green and Prussian blue depending on the concentration of the reducing agent. The presence of reducing agents acting as antioxidants in the samples causes the Fe^{3+}/ferricyanide complex to be reduced to the ferric form. Thus, Fe^{2+} can be tracked by measuring the formation of Prussian blue of Perl at 700 nm (Gülçin *et al.,* 2006). The absorbance values of oil samples and reference antioxidant substances at different concentrations at 700 nm were presented in Table 3. The higher the absorbance measured at 700 nm, the higher the reducing power. The data in Table 3 revealed that BHA had the highest FRAP value at 1000 ug/ml concentration, followed by BHT, Turmeric, and ginger, respectively. The Frap values of turmeric and ginger were almost close to each other. However, the absorbance value measured at 700 nm increased depending on the concentration.

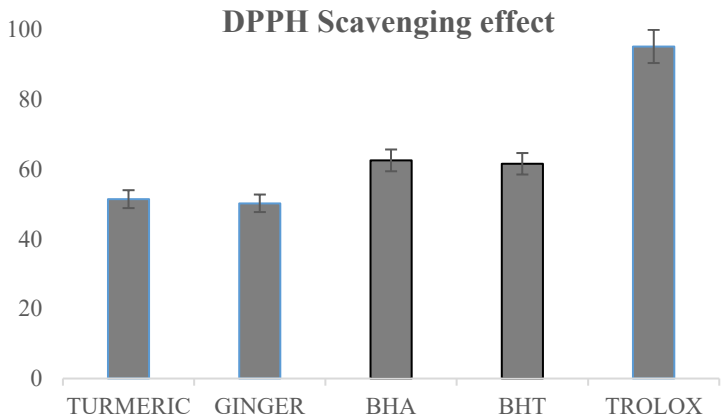
Table 3. Total reducing power of different concentrations (500–1000 µg/mL) of oil samples, BHA and BHT determined by Ferriccyanide method of the Fe^{3+}–Fe^{2+} transformation.

Sample	*FRAP value (at 700 nm)*	
	500 µg/mL	1000 µg/mL
BHA	2.834 ± 0.071*	3.029 ± 0.049
BHT	0.993 ± 0.0103	1.844 ± 0.058
TURMERIC	0.147 ± 0.013	0.351 ± 0.020
GINGER	0.131 ± 0.003	0.296 ± 0.007

* Data expressed as mean± S.D (n=3).

In this study, free radical scavenging activities of oil samples and standards such as BHA, BHT, and Trolox were determined using a DPPH method. DPPH is often used to evaluate different antioxidant substances' free radical scavenging effects (Erdoğan & Gökçe, 2021). When a DPPH solution is mixed with a substance that donates a hydrogen atom, this leads to the reduced form with loss of this violet color (Molyneux, 2003). Figure 2 displayed a significant decrease in the concentration of DPPH radical due to the scavenging ability of oil samples and standards. The scavenging effect of turmeric, ginger and standards on the DPPH radical decreased in the order of Trolox>BHA >BHT>Turmeric>Ginger which were 95.25 ± 0.05%, 62.57 ± 0.34%, 61.6 ± 0.3%, 51.45 ± 0.59%, and 50.26 ± 0.09%, at the concentration of 150µg/mL, respectively.

Figure 2. Scavenging effect of Turmeric, Ginger, BHA, BHT, and Trolox on the stable DPPH• at concentration 150 µg/mL. (DPPH•: 1,1-diphenyl-2-picryl-hydrazyl free radicals, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene, Data expressed as mean± *S.D* (n=3).



4. CONCLUSION

GC-MS essential oil analysis results from ginger and turmeric revealed that the oils were rich in sesquiterpene content. Both essential oils exhibited a strong antioxidant capacity. These essays have significant applications for the food and pharmaceutical industry. Moreover, the components used in the pharmaceutical, food, and cosmetics industries have also been identified in the essential oils of *C. Longa and Z. officinale*. These data revealed that turmeric and ginger profiles were similar in essential oil components and antioxidant capacity. This study also presented the total antioxidant capacity of pure essential oils of turmeric and ginger for the first time according to the CUPRAC method.

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The analyses in this study were carried out in the Department of Field Crops laboratory at the University of Applied Sciences in Isparta.

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.

Authorship contribution statement

Umit Erdogan: Writing-Original draft preparation, Formal Analysis, Conceptualization, Resources, Investigation, Supervision, Methodology, and Validation.

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Research Article

Antiproliferative and antioxidant potential of methanol extracts of aerial parts of *Colchicum boissieri* and *Colchicum balansae*

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Abstract: The antiproliferative and antioxidant activities, and total phenolic, flavonoid, and tannin contents of methanol extracts obtained from the aerial parts of *Colchicum boissieri* and *Colchicum balansae* were investigated in the present study. The evaluation of the antiproliferative effects of the extracts under study was carried out using the MTT assay. The antioxidant potentials of the extracts were assigned by using several assays (FRAP, CUPRAC, DPPH, ABTS, and *β*-carotene). The antiproliferative effects of both extracts on Caco-2 cells appeared to be generally similar for the incubation times tested. The antioxidant potential was found to be higher in the extract of *C. boissieri* (FRAP: on average 1.39 mg TE/g, CUPRAC: on average 10.06 mg TE/g, DPPH: on average IC_{50}= 0.59 mg/mL, ABTS: on average IC_{50}= 0.267 mg/mL, *β*-carotene: on average 78.58%) than the extract of *C. balansae*. In terms of total phenolic, flavonoid and tannins contents, *C. boissieri* extract (on average 1.97 mg GAE/g, 8.65 mg QE/g and 4.75 mg CE/g, respectively) was determined to be richer than *C. balansae* extract. The results suggest that both extracts have some biological properties for pharmaceutical applications. Further studies may contribute to the use of these plants for various purposes such as natural antioxidant sources or cancer agents.

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1. INTRODUCTION

Cancer is a multifactorial disease that has specific features at different levels as cellular, tissular, and organismic (Paul, 2020). Cancer is one of the leading causes of death all over the world. According to Sung *et al,* (2021), female breast cancer is the most diagnosed cancer followed by lung, colorectal, prostate, and stomach cancer. In addition, lung cancer continues to be the leading cause of cancer death followed by colorectal, liver, stomach, and female breast cancers. Many anticancer drugs were reported to have a narrow therapeutic potential because of their systemic toxicity and lack of selective properties against tumor (Kratz *et al.,* 2008).

Free radicals are generated by various metabolic processes and the uncontrolled or increased formation of free radicals in the body may lead to oxidative stress (Alkadi, 2020). The balance between free radicals and antioxidants are important for health (Lobo *et al.*2010). Oxidative

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stress has been known to contribute to various diseases such as cancer, atherosclerosis, diabetes, neurological disorders, and hypertension (Birben *et al.,* 2012). Antioxidants have the ability to neutralize free radicals and supply protection against damage owing to these free radicals (Zehiroglu & Sarikaya, 2019). Not only do plants have the potential for preventing or treating diseases but also they are of great interest as a good source for discovering new pharmaceutical drugs (Parasuraman, 2018). Plants contain secondary metabolites with various biological activities such as anti-inflammatory and anticancer, and have an important place in obtaining products with fewer side effects and higher efficiency in cancer treatment. In this context, screening of crude extracts of plants is important to provide natural agents with anticancer properties (Kooti *et al.,* 2017). In addition, plants or plant-based products, such as vegetables, flowers, fruits, cereals, and spices, have been known as the source of natural antioxidants. Considering their effects on human health, the evaluation of the antioxidant potentials of plants for different applications, including food additives, remains in interest (Xu *et al.,* 2017; Lourenço *et al.,* 2019).

Turkey is rich in terms of the *Colchicum* L. species it possesses. *Colchicum* species that are one of the significant plants with medicinal properties belong to Colchicaceae family and are evaluated in two groups in terms of flowering time in autumn or in spring. *Colchicum* genus contains species which are rich in alkaloids, and colchicine is the main alkaloid of this genus. Colchicine has been reported to have anticancer and anti-inflammatory effects, to be important in the treatment of several diseases such as gout, Familial Mediterranean Fever, psoriasis and other dermatological disorder, and also have a limited therapeutic index (Al-Snafi, 2016; Toplan *et al.,* 2016).

There have been studies on antioxidant activities of the extracts of the *C. boissieri* Orph. and *C. balansae* Planch. (Mammadov *et al.,* 2009; Sevim *et al.,* 2010), which are autumn-flowering species from Turkey (Toplan *et al.,* 2016). However, no literature is available about the effects of methanol extracts of aerial parts of plants in question on the cell viability of Caco-2 (human colorectal adenocarcinoma) cells. The present study, therefore, aims to contribute to the literature about antiproliferative effects against colorectal human cancer cell line, and also antioxidant properties as well as the amount of total phenolic, flavonoid and tannin contents of their methanolic aerial part extracts.

2. MATERIAL and METHODS

2.1. Chemicals and Reagents

The chemicals and reagents were provided from Biochrom (GmbH, Germany), Capricorn Scientific (GmbH, Germany), and Applichem (Germany) for cell culture assay and from Sigma-Aldrich (Germany) for other experiments.

2.2. Plant Materials and Preparation of the Plant Extracts

The aerial parts of *Colchicum boissieri* (1400 m, Yılanlı Mountain) and *Colchicum balansae* (30 m, Ula) were obtained from the Muğla province, Turkey in October 2021. An expert taxonomist Dr. Olcay Ceylan at Muğla Sıtkı Koçman University, Muğla, Turkey, carried out authentication of the plant specimens. Herbarium specimens of these plants were kept in the herbarium of Biology Department at Muğla Sıtkı Koçman University. After the plant parts dried in shadow at room temperature were milled using a blender, about 10 g of plant sample was soaked in 100 mL of methanol, and then shaked at 55°C for 24 h. Each extract solution was filtered, followed by the removal of methanol using a rotary evaporator (IKA RV10D, Staufen, Germany). After lyophilization, the extracts obtained were deposited at -20°C in the dark (Turan & Mammadov, 2018).

2.3. Cell Culture

Colorectal (Caco-2, ATCC) human cancer cell line was grown in Roswell Park Memorial Institute (RPMI)-1640 medium which was supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured at 37°C in a humidified incubator with 5% CO_2 and monitored daily using an inverted microscope (Zeiss, Germany).

2.4. MTT Assay

The antiproliferative activities of the plant extracts were assigned based on MTT assay (Mosmann, 1983) with some modifications for 24 h and 48 h. Plant extracts were separately dissolved in 10% DMSO (Dimethyl sulfoxide) to obtain stock solution and diluted with cell growth medium before addition to the cells. The seven serial dilutions of the extracts (0.02-1.2 mg/mL) were used in this assay. To detect the cell viability, the cells at $2x10^4$ cells/well were seeded into 96 well plates. After incubation at 37°C for 24 h, the cells were exposed to different concentrations of plant extracts for 24 h and 48 h. Cells not exposed to any extract were control cells. After each incubation time, the medium in each well was replaced by 100 µL of fresh growth medium and 10 µL of MTT (5 mg/mL) was added to each well, followed by 4 h incubation. 100 µL of DMSO were added to each well after removing the medium. The microplates were shaken at 150 rpm for 6 min and the absorbance was recorded at 540 nm using a microplate reader (ThermoScientific, Multiscan FC, USA). Cell viability was expressed as a percentage cell viability by considering the absorbance of the treated and control cells.

2.5. Antioxidant Potential of the Plant Extracts

Ferric ion reducing antioxidant power (FRAP) assay and Cupric ion reducing antioxidant capacity (CUPRAC) assay were done based on the previous methods of Benzie and Strain (1996) and Apak *et al*. (2006), respectively and the outcomes were expressed as milligram of Trolox equivalent per gram of the extract (mgTE/g). For the investigation of DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity of the extracts, the methods stated by Turan and Mammadov (2018) and Re *et al.* (1999) were followed, respectively, and the results are presented as IC_{50} values. *β*-carotene–linoleic acid method used for evaluation of the antioxidant capacity of the extracts was conducted taking into account the method described by Amarowicz *et al.* (2004), and also the total antioxidant activity was calculated according to the formula of Amarowicz *et al. (*2004) and expressed as a percentage.

2.6. Total Phenolic, Flavonoid and Tannin Contents

The total phenolic, total flavonoid, and total tannin contents of the extracts were evaluated by referring to the methods described by Turan and Mammadov (2018), Aryal *et al*. (2019), and Bekir *et al.* (2013). The results are presented as equivalents of Gallic acid (mgGAE/g), Quercetin (mgQE/g), and Catechin (mgCE/g) in milligrams per g of the extract, respectively.

2.7. Data Analysis

The SPSS software (version 22.0 for Windows, IBM Corp., Armonk, N.Y., USA) was used to evaluate the results and compute the IC_{50} (half maximal inhibitory concentration) values. The results obtained from at least three separate experiments were expressed as mean ± SE (Standard Error). The statistical analyses of data were done with ANOVA (Analysis of Variance) and then Tukey multiple comparison test ($p<0.05$). A t-test was used when there were only two groups. The graphs were presented using the GraphPad Prism 7.0 Software program.

3. RESULTS and DISCUSSION

3.1. Antiproliferative Activity of Plant Extracts

MTT assay, which is basically based on the conversion of MTT into insoluble purple-colored formazan crystals by viable cells, is one of the commonly used colorimetric assays. In addition, DMSO is one of the solvents used to dissolve the formazan crystals before recording the absorbance (Kamiloglu *et al.,* 2020). The effects of the extracts on the viability of Caco-2 cells were determined using MTT assay and the results are shown in Figure 1 and IC_{50} values are also presented in Table 1. Given these results, the antiproliferative effects of both extracts on Caco-2 cells appear similar.

Figure 1. Antiproliferative activity of the *Colchicum boissieri* (a) and *Colchicum balansae* (b) extracts on Caco-2 cell line. Statistical analysis for results of MTT assay at each incubation time was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

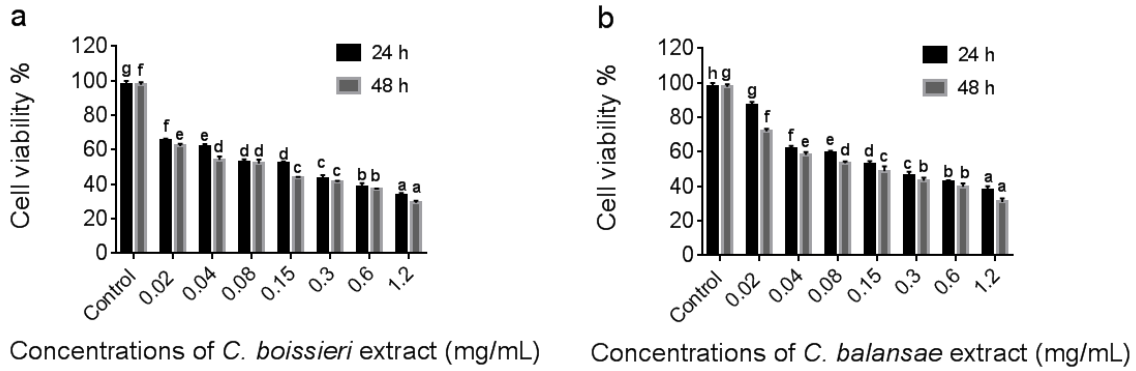

Table 1. Approximate IC_{50} values of the *Colchicum boissieri* and *Colchicum balansae* extracts

	IC_{50} values (mg/mL)	
Plant extracts	24 h	48 h
C. boissieri	0.2	0.1
C. balansae	0.217	0.131

Pırıldar *et al.* (2010) reported that all extracts, except for the seed extract, exhibited more cytotoxic activity against the promyeloid leukemia cell line (HL60) than against the chronic myeloid leukemia cell line (K562), among methanol extracts obtained from the different parts of *C. baytopiorum* CD Brickell. The highest cytotoxic activity observed on HeLa (cervical cancer) cell line was declared to originate from the methanolic extract of cormus of *C. sanguicolle* K.M. Perss among different plant extracts tested (Artun *et al.,* 2016). Becer *et al.* (2019) stated that the extract of *C. pusillum* Sieber at all concentrations tested for 24 h and 48 h caused the toxic effect against Colo-320 (human colon adenocarcinoma) cell in a dose and time-dependent manner. Also, other previous studies reported the antiproliferative activity of the extracts obtained from different *Colchicum* species (Ozsoylemez *et al.,* 2016; Gulsoy-Toplan *et al.,* 2018; Dagdeviren Ozsoylemez & Ozcan, 2021). However, according to available literature, the present study is most likely the first study based on the antiproliferative effects of the *C. boissieri* and *C. balansae* extract on Caco-2 cell line.

3.2. Antioxidant capacity of plant extracts

Antioxidant activities of the methanol extract of aerial parts of *C. boissieri* and *C. balansae* were evaluated by different assays and the results are shown in Table 2. The antioxidant activity of the extract of *C. boissieri* was determined to be better than the extract of *C. balansae*. Considering the reducing potential, FRAP, and CUPRAC activities for *C. boissieri* extract were 1.39±0.03 and 10.06±0.73 mg TE/g, respectively. In addition, according to the results of DPPH,

ABTS and β-carotene/linoleic acid assay, the antioxidant activities of both extracts were low compared to that of BHA (Butylated hydroxyanisole) used as standard. The IC_{50} values of DPPH and ABTS radical scavenging tests for *C. boissieri* extract were 0.59±0.018 and 0.267±0.008 mg/mL, respectively. Also, the highest antioxidant activity between the plant extracts was detected as 78.58±4.64% for *C. boissieri* extract using the β-carotene/linoleic acid method.

Table 2. Antioxidant activity of *Colchicum boissieri* and *Colchicum balansae* extracts.

Plant extracts	FRAP (mg TE/g)	CUPRAC (mg TE/g)	DPPH (IC_{50}, mg/mL)	ABTS (IC_{50}, mg/mL)	β-carotene/ linoleic acid (%)
C. boissieri	1.39±0.03a	10.06±0.73a	0.59±0.018b	0.267±0.008b	78.58±4.64b
C. balansae	0.65±0.03b	3.59±0.58b	1.43±0.049c	0.487±0.095c	69.58±3.81c
BHA	nt	nt	0.019±0.003a	0.008±0.004a	88.41 ±0.36a

The values are presented as mean ± standard error (SE). The different lowercase letters in the same column indicate significant differences. nt: not tested

Antioxidants are capable of decreasing oxidative stress, therefore they have a critical function in stopping and curing ailments, sustaining health. Many methods have been used for the analysis of antioxidant activity (Munteanu and Apetrei, 2021). In the present study, the different assays; namely, FRAP, CUPRAC, DPPH, ABTS, and the β-carotene/linoleic acid method were conducted for the assessment of the antioxidant potential of the extracts.

DPPH and ABTS scavenging assays are commonly used simple spectrophotometric methods and the antioxidant activity is determined colorimetrically based on absorbance changes of stable colored radicals. The FRAP and CUPRAC assays, which are spectrophotometric methods, determine the capacity of an antioxidant to reduce an oxidant that changes colorimetrically when reduction occurs. The principles of FRAP and CUPRAC assays are the reduction of Fe^{3+}-ligand complex to Fe^{2+} and reduction of Cu^{2+} to Cu^{+}, respectively, by means of antioxidants (Pisoschi & Negulescu, 2011; Munteanu & Apetrei, 2021). Another common assay for the evaluation of the antioxidant activity of the plant extracts is the β-carotene bleaching assay. Briefly, the discoloration of β-carotene is observed due to radical species originating from the oxidation of linoleic acid and the presence of antioxidants can be delayed discoloration and the bleaching the color is spectrophotometrically measured with ease (Pisoschi & Negulescu, 2011; Xiao *et al.,* 2020). As a result of the assays aforementioned, the *C. boissieri* extract caused a higher antioxidant activity than that of *C. balansae* extract. In a previous study, the antioxidant activities of the extracts from tuber and leaf of *C. balansae* from Muğla province by using different solvents were examined using β-carotene bleaching method and DPPH scavenging activity assay. In the same study, the leaves extracts of *C. balansae* displayed higher antioxidant properties than those of tuber extract, and also the highest antioxidant activity efficiency and the highest free radical scavenging activity were detected in leaf ethanol (%64) and leaf benzene (68.35%) extract, respectively (Mammadov *et al.,* 2009). Unlike the current study, Sevim *et al.* (2010) declared that the DPPH scavenging activities of the methanol extracts from *Colchicum* taxon including *C. boissieri* and *C. balansae* tested by them were to be low below 40% at 2000 µg/mL. The antioxidant capacities of flower, root, and leaf extracts obtained from *C. szovitsii* subsp. *szovitsii* (Rocchetti *et al*., 2019) and *C. triphyllum* (Senizza *et al.,* 2020) obtained using different extraction procedures were higher than those in the present study in terms of FRAP and CUPRAC assays. The antioxidant activities of the extract of different *Colchicum species* for example *C. speciosum* Steven (Souri *et al.,* 2008), *C. turcicum* Janka (Kiliç *et al.,* 2014), *C. autumnale* L. (Suica-Bunghez *et al.,* 2017; Hailu *et al.,* 2021) were also examined.

3.3. Total Bioactive Compounds of Plant Extracts

The methanolic aerial part extract of *C. boissieri* was found to be richer than *C. balansae* extract in terms of total phenolic, flavonoid, and tannins contents (Table 3), with 1.97±0,07 mg GAE/g, 8.65±1.67 mg QE/g and 4.75±0.51 mg CE/g, respectively.

Table 3. Total phenolic, total flavonoid, and total tannin contents of *C. boissieri* and *C. balansae* extracts

Plant extracts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	Total tannin content (mg CE/g)
C. boissieri	1.97±0.07a	8.65±1.67a	4.75±0.51a
C. balansae	1.05±0.05b	5.37±0.11a	3.00±0.24b

The values are presented as mean ± standart error (SE). The different lowercase letters in the same column indicate significant differences.

Natural antioxidants of plant origin such as phenolic acids, flavonoids, lignans, and tannins have been reported to show various biological activities such as antibacterial, antiaging, anticancer, anti-inflammatory, and antioxidant (Xu *et al.,* 2017). The higher antioxidant potential of *C. boissieri* extract may be attributed to the presence of bioactive compounds tested and found to be higher than that of *C. balansae* extract. The total phenolic contents of *C. boissieri* and *C. balansae* extract tested here were found to be lower than the previously reported value by Rocchetti *et al.* (2019) who determined that for the flower extracts, the highest total phenolic content was found to be in the aqueous macerated extract of flower of *C. szovitsii* Fisch. & C.A.Mey. subsp. *szovitsii* (40.70 mgGAE/g). The total flavonoid contents of water and acetone extract of *C. turcicum* (Kiliç *et al*., 2014) were recorded as 13.1 mg GAE/g and 83.9 mg GAE/g, respectively, and also the total phenolic contents of their extracts in question were expressed as catechol equivalents and varied from 0.454 mg CE/g (water) to 2.172 mg CE/g (acetone). Many researchers have also revealed the amount of the total bioactive contents of various *Colchicum* species extract; for example, C. *kurdicum* (Bornm.) Stef. (Azadbakht *et al.,* 2020), *C. autumnale (*Suica-Bunghez *et al*., 2017; Hailu *et al*., 2021), *C. speciosum*, C. *robustum* Stef. (Davoodi *et al*., 2021).

4. CONCLUSION

The results of the present study revealed that the extract of *C. boissieri* has more potential than that of *C. balansae* in terms of antioxidant activity. In addition, total bioactive contents evaluated are higher in the extract of *C. boissieri* than in the extract of *C. balansae*. The antiproliferative effects of both extracts on Caco-2 cells were generally observed to appear similarly. The current study is most likely the first study based on the total bioactive contents of extracts, especially *C. boissieri* and the antiproliferative effects of them on the Caco-2 cell line. Of the extracts assessed, *C. boisserie* is a relatively promising nominee for future investigations compared to *C. balansae*. Various biological studies to be carried out in future related to these species, including isolation and identification of their phytochemicals, may contribute to their application such as food additives, sources of natural antioxidants, and anticancer agents.

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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(s). Ethics Committee Approval and its number should be given by stating the institution name which gave the ethical approval.

Authorship contribution statement

Mehlika Alper: Investigation, Analysis, and Writing - original draft

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Research Article

Essential oil composition of two endemic *Nepeta* L. (Lamiaceae) taxa from Southwestern Turkey

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Abstract: *Nepeta* L. is one of the important genus in the Lamiaceae family. It includes *ca.* 300 herbaceous species and mostly grows in Eurasia. *Nepeta* is represented in Turkey by 40 taxa and of these 16 are endemic. *Nepeta* species are commonly utilized in traditional medicine by the local people, primarily as spasmolytic, diuretic, and bronchodilator agents. As a consequence of studies on *Nepeta* taxa, terpenoids and flavonoids have been identified as the most common components. In this report, chemical contents of two endemic *Nepeta* taxa (*N. viscida* from Buharkent/Aydın and *N. nuda* L. subsp. *lydiae* from Altınyayla/Burdur) were presented. The main constituents were determined as *α*-terpineol (20.59%), *trans*-*β*-caryophyllene (9.90%) and spathulenol (9.37%) for *N. viscida,* and 1,8-cineole (31.31%), borneol (18.95%) and caryophyllene oxide (14.59%) for *N. nuda* subsp. *lydiae.*

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1. INTRODUCTION

The Lamiaceae (=Labiatae) is a family in the Lamiales order, with 236 genera and over 7000 species (El Khoury *et al.,* 2019). At this point, 226 genera have been allocated to seven subfamilies, with ten genera classified as *incertae sedis* since they could not be assigned to any of the subfamilies (Harley *et al.,* 2004; Jamzad, 2013). In addition, five new subfamilies have been defined recently (Li *et al.,* 2016; Li & Olmstead, 2017). Lamiaceae family is the third-largest family in the Flora of Turkey in terms of the number of taxa (Celep & Dirmenci, 2017), and the endemism rate is approximately 44% in this area (Baser & Kırımer, 2018). Turkey is recognized as a major gene center for the Lamiaceae (Celep & Dirmenci, 2017; Baser & Kırımer, 2018). Lamiaceae members are characterized by their strong aromatic properties such as rich essential oil contents. Humans have probably used these plants since ancient times. Archeological evidence suggests that Lamiaceae members were once cultivated on a local scale (Nuńez & De Castro, 1992; Rattray & Van Wyk, 2021). Today, *Mentha*, *Thymus*, *Origanum*, *Salvia*, and *Nepeta* species are frequently used in traditional and modern medicine almost all over the world (Naghibi *et al.,* 2005).

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The *Nepeta* L. is one of the richest genera among the members of the Lamiaceae family in Turkey in terms of the number of taxa. It is a member of the tribe *Mentheae* (subfamily *Nepetoideae*) and includes about 300 species (Asgarpanah *et al.,* 2014). In Turkey, there are 40 taxa in the *Nepeta* genus, 16 of which are endemic (Gökbulut & Yılmaz, 2020). *Nepeta* species are commonly utilized in traditional medicine by the local people, primarily as spasmolytic, diuretic and bronchodilator agents (Sharma *et al.,* 2021). As a consequence of studies on *Nepeta* species, terpenoids and flavonoids have been identified as the most common components (Sharma *et al.,* 2021). Several *Nepeta* species have long been recognized to offer feline attractant characteristics. The feline attractant action of the genus is thought to be caused by nepetalactone and its isomers (Baser *et al.,* 2000). The first phytochemical research on *Nepeta* species dates back to 1955 (McElvain & Eisenbraun, 1955). Since then, various chemical compounds have been reported within the genus. Until 2010, *ca.* 200 compounds have been recognized from *Nepeta* species (Formisano *et al.,* 2011). Some societies have learned to use *Nepeta* species primarily for flavor as well as medical purposes such as venereal diseases, aphrodisiac, headaches, backaches, rheumatic pain, sunburn, diuretic, wound healing agents (Koyuncu *e et al.,* 2010; Mükemre *et al.,* 2015; Gomes *et al.,* 2020).

The literature searches indicated that the chemical profiles of the essential oil of some other *Nepeta* species have previously been studied (Baser *et al.,* 1993-1995-1998-2000-2001; Baser & Özek, 1994; Kökdil *et al.,* 1996-1997-1998; Tümen *et al.,* 1999; Senatore & Özcan, 2003; Tepe *et al.,* 2007; Emre *et al.,* 2011; İşcan *et al.,* 2011; Kilic *et al.,* 2011-2013; Gormez *et al.,* 2013; Bozok *et al.,* 2017; Bozok, 2018; Sarıkurkcu *et al.,* 2018; Akdeniz *et al.,* 2020; Karakus *et al.,* 2021; Zengin *et al.,* 2021), but there is no report for the essential oil contents of *N. viscida* Boiss. and *N. nuda* L. subsp. *lydiae* P.H. Davis in the localities used in our study. In this report, the chemical contents of the essential oil of two endemic *Nepeta* taxa were presented.

2. MATERIAL and METHODS

2.1. Plant Materials and Sample Preparation

Nepeta viscida and *N. nuda* subsp. *lydiae* were sampled at their flowering period from their natural habitats (Buharkent, Aydın-Turkey and Altınyayla, Burdur-Turkey, respectively). The collected species were identified by Prof. Dr. Gürkan SEMİZ and voucher specimens (GSE2020 for *Nepeta viscida* and GSE2004 for *N. nuda* L. subsp. *lydiae*) were deposited in the Chemical Ecology Laboratory Herbarium of the Pamukkale University, Biology Department in Denizli, Turkey. The air-dried aerial parts of each species (100 mg) were cut into small pieces and powdered. The essential oils were collected using a Clevenger-type apparatus with hydro-distillation for 4 hours. The essential oils were stored in amber bottles at 4°C until analysis.

2.2. GC-MS Analysis

The chemical profiles of the essential oils were analyzed on Gas Chromatography-Mass Spectrometer (Hewlett Packard GC-7820A, MSD-5975). A 30 m-long HP-5MS capillary column was used (ID 0.25 mm, film thickness 0.25 mm, Hewlett Packard). The chromatographic conditions to obtain for mono- and sesquiterpenes were followed by Semiz *et al* (2018). The percentages were calculated from the GC peak areas using the normalization procedure.

3. RESULTS

In this study, the essential oils of *N. viscida* and *N. nuda* L. subsp. *lydiae* were characterized by GC-MS. Chromatographic analysis of the essential oils showed that the chemical compositions of *N. viscida* and *N. nuda* subsp. *lydiae* were more or less similar to each other but differed between the amounts of the compounds. The essential oil contents of our *Nepeta* species were

dominated by mainly monoterpene and sesquiterpenes hydrocarbons. Essential oil yield was found as 0.12% for *N. viscida* and 0.08% for *N. nuda* subsp. *lydiae* based on the dry weights.

Table 1. Essential oil composition (%) of *N. nuda* subsp. *lydiae* and *N. Viscida.*

No	RRI*	Compounds	*N. nuda* subsp. *lydiae*	*N. viscida*
1	922	tricyclene	0.20	-
2	937	*α*-pinene	0.41	0.19
3	969	sabinene	-	0.36
4	974	*β*-pinene	0.10	1.91
5	983	myrcene	0.20	0.32
6	1009	3-carene	1.72	-
7	1020	*p*-cymene	0.71	-
8	1028	1,8-cineol	**30.90**	4.60
9	1032	limonene	-	0.36
10	1035	*β*-ocimene	1.22	-
11	1052	*γ*-terpinene	0.71	-
12	1090	linalool	10.78	2.88
13	1134	*trans*-pinocarveol	-	0.19
14	1142	camphor	2.23	-
15	1160	borneol	**18.70**	-
16	1164	*δ*-terpineol	2.13	-
17	1176	terpinen-4-ol	0.81	0.95
18	1186	*α*-terpineol	-	**20.59**
19	1204	verbenone	0.20	-
20	1230	pulegone	1.01	-
21	1252	geraniol	1.82	-
22	1336	bicycloelemene	-	0.81
23	1357	*α*-cubebene	-	0.15
24	1376	*α*-copaene	-	0.90
25	1382	*β*-cubebene	-	0.24
26	1383	*β*-bourbonene	1.22	-
27	1392	*β*-elemene	0.71	0.93
28	1406	*α*-gurjunene	-	3.03
29	1411	*trans*-*β*-caryophyllene	-	**9.90**
30	1438	aromadendrene	-	0.74
31	1448	*trans*-*β*-farnesene	4.36	2.46
32	1453	*α*-humulene	0.81	4.32
33	1474	germacrene-D	1.52	1.92
34	1490	zingiberene	-	3.64
35	1499	*β*-bisabolene	-	2.08
36	1505	*γ*-cadinene	-	0.62
37	1512	*δ*-cadinene	0.41	4.88
38	1548	germacrene-B	-	6.38
39	1564	palustrol	-	2.92
40	1570	spathulenol	-	**9.37**
41	1578	caryophyllene oxide	**14.40**	2.36
42	1590	viridiflorol	-	0.56
43	1602	ledol	-	3.19
44	1641	*α*-cadinol	-	2.28
45	1710	farnesol	1.42	-

[a] Compounds listed in order their elution,
[b] RRI: Relative retention indices measured to against *n*-alkanes on HP-5MS column,
[c] The values in bold indicate the highest amounts.

Thirty-two compounds representing 96.0% of total oil were detected in *N. viscida*, and twenty-five compounds representing 98.7% of the total oil were detected in *N. nuda* subsp. *lydiae*. The percentage compositions of the essential oils were listed in Table 1. The main constituents were determined as *α*-terpineol (11.78%), *trans-β*-caryophyllene (5.66%) and spathulenol (5.36%) for *N. viscida*, and 1,8-cineole (31.31%), borneol (18.95%) and caryophyllene oxide (14.59%) for *N. nuda* subsp. *lydiae.*

4. DISCUSSION and CONCLUSION

The composition of main components in *Nepeta* species' essential oils has been categorized into two groups. Group I contains some isomers of nepetalactone, whereas Group II contains compounds other than nepetalactone isomers as main components, such as 1,8-cineole, *β*-caryophyllene, caryophyllene oxide (Sharma & Cannoo, 2013). At this point, our *Nepeta* species should be classified in Group II because of the most abundant components (1,8-cineole for *N. nuda* subsp. *lydiae* and *α*-terpineol for *N. viscida*). However, in a previous study by Kabalay *et al* (2018), they showed that neptalactone isomers were the most abundant compound for *N. nuda* subsp. *lydiae*. The plant samples in their study were collected from a different locality compared to our study. There are almost no studies in the literature, except for Kabalay *et al* (2018), which revealed the chemical composition of the essential oil of *N. nuda* subsp. *lydiae*. Therefore, our study is the most detailed chemical content study for *N. nuda* subsp. *lydiae* in the current literature.

Baser *et al* (1995) showed that the most abundant component of the essential oil of *N. viscida* from Manisa region was found as *α*-terpineol. In another study, Carikci (2021) evaluated the essential oils contents of *N. viscida* from two different localities from Balikesir and İzmir, and spathulenol and 1,8-cineole were the most abundant components, respectively. The results of our study are partially similar to the results of these studies. The differences in composition could be explained by the chemotype, soil factors, climatic conditions or geographic location. It was clearly explained that the production of secondary compounds can be affected by climate drivers (Tingey *et al.,* 1980; Banthorpe & Njar, 1984; Kainulainen *et al.,* 1992; Loziené *et al.,* 2008; Blanch *et al.,* 2009; Ormeño & Fernandez, 2012; Yu *et al.,* 2021). *Nepeta* species have been associated with several medicinal benefits since ancient times. Scientists have only recently become aware of its new potential therapeutic properties (Baytop, 1999). Significant scientific advances in the chemical compositions and bioactivities of *Nepeta* species from Turkey have been declared (Baser *et al.,* 1993-1995-1998-2000-2001; Baser & Özek, 1994; Kökdil *et al.,* 1996-1997-1998; Tümen *et al.,* 1999; Senatore & Özcan, 2003; Tepe *et al.,* 2007; Emre *et al.,* 2011; İşcan *et al.,* 2011; Kilic *et al.,* 2011-2013; Gormez *et al.,* 2013; Bozok *et al.,* 2017; Bozok, 2018; Sarıkurkcu *et al.,* 2018; Akdeniz *et al.,* 2020; Karakus *et al.,* 2021; Zengin *et al.,* 2021). As a conclusion, we believe that our results will encourage more investigation into the chemistry of *Nepeta* species and chemical content profiling of the species using terpenes may be useful in taxonomical studies.

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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

Gurkan Semiz: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing-original draft. **Batikan Gunal**: Methodology, Supervision, and Validation. **Metin Armagan.** Investigation, Supervision, Writing – original draft.

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Research Article

Comprehensive Study on BeeBread: Palynological Analysis, Chemical Composition, Antioxidant and Cytotoxic Activities

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Abstract: Bee bread is a bee product obtained as a result of fermentation of pollen stored by honey bees in the cells of the honeycomb. Palynological analysis, chemical composition, antioxidant activity of bee bread and its cytotoxic effect against human lung carcinoma (A549), human prostate cancer (DU 145) and human neuroblastoma (SH-SY5Y) cell lines were investigated in this study. 25 plant taxa were identified with palynological analysis. Fatty acids, cyclic, aromatic, phenolic, terpenoid, diterpen and metallic complex structures were seen in GC-MS results. FTIR consequence were compatible with GC-MS results and the structure types of FTIR results were seen in the dominant compounds of GC-MS results. Radical scavenging activity (RSA) of bee bread showed inhibition variability between 20.15 ± 0.68% and 93.18 ± 0.44% depending on the concentration. In addition, the EC50 value was measured as 80.08 ± 0.10 mg/mL. Bee bread exhibited moderately cytotoxic effect at all concentrations (15.625 - 2000 µg/mL) against A549, DU 145, and SH-SY5Y cell lines. Bee bread can be used in medical fields because of it's antioxidant and anticancer properties.

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1. INTRODUCTION

Honey bees collect pollen, nectar and water to meet their nutritional requirements (Gilliam, 1979). Pollen grains have the male reproductive cell of seed plants. It contains most of the nutrients necessary for the development of young worker bees and larvae (Liu *et al.*, 2015). The pollen pellets brought by the worker bees are filled into the honeycomb cells. The stored pollen grains are covered with honey and beeswax, undergoing a chemical change. Thereafter this chemical change, bee pollen turns into the bee bread (Gilliam, 1979). Bee bread is produced by

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lactic acid fermentation of honey bee secretions, honey and bee pollen (Mărgăoan *et al.*, 2019). Adult honey bees and their larvae are fed with bee bread (Gilliam, 1979), which has beneficial effects on human health. Bee bread is among the functional foods because it contains bioactive compounds like omega fatty acids, essential amino acids, proteins, lipid, vitamin, mineral, and simple sugars (Bobiş *et al.*, 2010; Mărgăoan *et al.*, 2019).

The composition of bee bread varies according to the botanical origin of pollen, but basically, its structure consists of water, lipids, carbohydrates, proteins, vitamins B and C, reproductive hormones, nucleic acids, inorganic elements, acetylcholine, decanoic acid, pantothenic acid, gamma globulin, neopterin, and biopterin etc. (Giroud *et al.*, 2013; Sobral *et al.*, 2017).

Cancer is one of the deadliest diseases in the world, despite many developed methods and therapeutic drugs. However, it is difficult to cure because the immune system is damaged in the treatment of tumors. Consequently, there is a need to unearth new anti-tumor molecules that strengthen the immune system without harming the patient (Yang *et al.*, 2007). Lung cancer is the most occurring type of cancer that is diagnosed in males, and causes deaths. Moreover, it is the fourth most common cancer in women. Lung cancer also ranks second in cancer-related deaths in women. In this type of cancer, 13% (1.6 million) cases were seen in 2008. It has been reported that 18% (1.4 million) of deaths same year are caused by this disease (Jemal *et al.*, 2011). Thereafter lung cancer, prostate cancer is also the second most occurring cancer diagnosed in men. It ranks sixth in causes of death among men (Jemal *et al.*, 2011; Tuzcu *et al.*, 2017). In 2012, it was reported that 8.2 million died from cancer of 14.1 million new cases worldwide. By 2030, it is predicted that the number of new cases and deaths from cancer will increase approximately to double (Ferlay *et al.*, 2015; Tuzcu *et al.*, 2017). Neuroblastoma is also the most common tumor among children under 1 year old. Every year, 700 cases in the USA and Canada and 1,500 cases in Europe are reported. This is approximately twenty-eight percent of all cancers diagnosed in European and US babies (Heck *et al.*, 2009). Anticancer activity of honey and propolis has been extensively studied in some cancer cells (Barbarić *et al.*, 2011; Borges *et al.*, 2011; da Silva Frozza *et al.*, 2013; Markiewicz-Żukowska *et al.*, 2013). There are not many studies on the anticancer effects of bee bread (Sobral *et al.*, 2017).

In this study, palynological analysis, chemical composition, and antioxidant activity of bee bread produced in Bingöl-Türkiye and its cytotoxic effect on some cancer cell lines were investigated. Studies haven't been conducted yet with bee bread on the cancer cell lines evaluated with this study. The aim of our study is to give an idea to the cancer research to be made with bee bread.

2. MATERIAL and METHODS

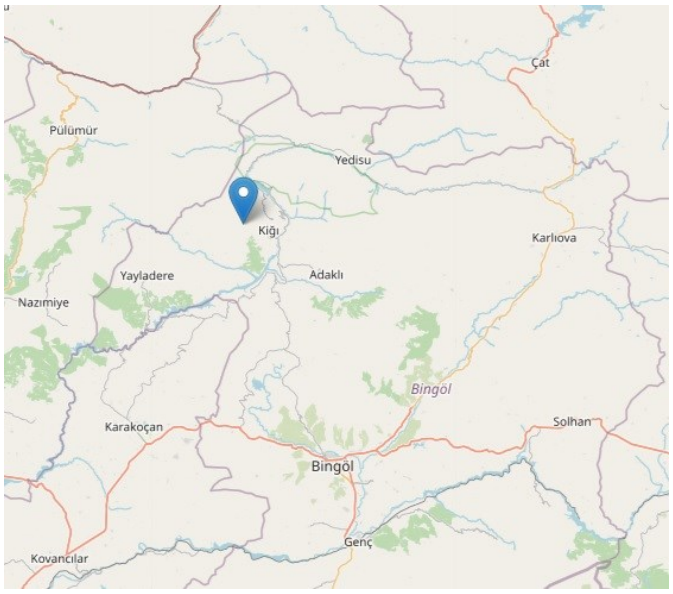
2.1. Bee Bread Sample

Bee bread was procured from beekeepers of the village Ölmez, in Kiğı Region, Bingöl Province, Türkiye (40° 17′ 21.9156"N - 39° 19′ 3.4032"E) in 2020 (Figure 1). The collected bee bread samples were stored at -20 °C until the bee bread extracts were prepared.

2.2. Chemicals and Reagents

Ethanol (~96% v/v) was procured from Alkomed Kimya Ltd. Sti., Türkiye. DMEM/F12 medium, fetal bovine serum (FBS) and penicillin-streptomycin solution were procured from Gibco Life Technologies, Paisley, UK. All other reagents and chemicals were purchased from Merck (Darmstadt, Germany).

Figure 1. A map giving the approximate location of procured bee bread sample.



2.3. Preparation of Bee Bread Extraction

Ethanolic extract of bee bread (EBB) was prepared at the rate of 10% (w/w) by using 96% ethanol (v/v), according to the method given by Markiewicz-Żukowska *et al.* (2013) with small modifications. Briefly, 10 g bee bread was immersed in a 90 g ethanol solution. The mixture was mixed for 24 hours with a magnetic stirrer at 25 °C and then was filtered by using Whatman no: 2 Cellulose Filter Paper (diameter: 125 mm). The filtrate was obtained and then evaporated at 40°C in a rotary evaporator (Rotavapor R-3, Buchi, Switzerland). The obtained residues were then stored in a refrigerator at -20°C for use in experimental analyses.

2.4. Palynological Analysis

Pollen analysis was performed with small modification by using the methods of Luz and Barth (2012). Bee bread pollen slides were prepared and investigated with Leica DM 2500 microscope. Minimum 500 pollen grains were counted on the slide. The pollen frequency percentages of the bee bread sample were indicated by using the methodology of Wróblewska *et al.* (2006). The pollen percentages were considered as <3% was sporadic group, 3-15% was minor group, 16-44% was secondary group, and ≥ 45% was dominant group.

2.5. Fourier-Transform Infrared Spectrometry (FTIR)

The properties of different chemical molecules and their organic bond structures in the EBB sample were determined via FTIR spectrometer. The analysis was carried out according to the study of ERGUN *et al.* (2017). FTIR spectrometer (Perkin-Elmer 100, Perkin-Elmer Inc., Norwalk, CT, USA) equipped with an attenuated total reflectance accessory (ATR; Perkin-Elmer) was used for acquiring spectra from EBB. The EBB sample was placed in the Diamond/ZnSe crystal cell. The sample was scanned with 4 cm^{-1} resolution for 5 scans in the wavenumber of 4000 – 650 cm^{-1}. EBB sample was read three times. For processing, the average spectrum within the sample was used. Spectrum 100 (version 6.3.5, 1999) and Spekwin32 (version 1.71.6.1, 2012) software were used for processing spectra of samples. The EBB data were statistically analyzed and compared utilizing Duncan multiple range test.

2.6. GC-MS Analysis

Chemical composition of EBB was determined with gas chromatography/mass spectrometry (GC-MS) analysis. The method was performed as described in previous published study of ÇAKIR *et al.* (2020).

2.7. Antioxidant Activity

The antioxidant activity of EBB was estimated with the $DPPH^+$ free radical scavenging assay, as described by Hatano *et al.* (1988); Kaya *et al.* (2018).

2.8. Cell Culture

Human prostate cancer DU 145 (ATCC: HTB-81), human neuroblastoma SH-SY5Y (ATCC: CRL-2266) and human lung carcinoma A549 (ATCC: CCL-185) cell lines were used in this study. The related cells were cultured in a Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (DMEM/F12) supplemented with 12% fetal bovine serum and 0.5% penicillin-streptomycin antibiotic solution. The cells were grown in an incubator at 37 °C with 5% CO_2 and subcultured every two or three days.

2.9. Cell Viability Assay

WST-1 cell proliferation and cytotoxicity assay kit (AR1159, Boster, China) was used to analyze the cytotoxic activity of EBB on the A549, DU 145, and SH-SY5Y tumor cell lines. The tumor cells were grown in *T25* flasks. The cells were harvested and then counted using a Thoma hemocytometer. 1×10^4 cells/well in a 100 µL medium were seeded in a 96-well plate. After 24 h, the cells were treated with different concentrations of EBB (15.625–2000 µg/mL). After 48 h, 5 µl of WST-1 reagent was added to each well and approximately 4 h later of incubation, the absorbance was measured at 450 nm using a microplate reader (SpectraMax Plus 384 Molecular Devices, USA).

2.10. Statistical Analysis

All measurements were repeated three times, and GraphPad Prism 5.01 software was applied for statistical analysis. Comparable datasets were assessed and the analyses were conducted by using two-tailed Student's t-test ($p<0.05$ was considered significant).

3. RESULTS and DISCUSSION

Plant source analysis of bee bread is quite limited. As a result of palynological analysis of bee bread, the pollen grains of 25 different taxa belonging to 13 different families were identified (Table 1) and belonged to Asteraceae, Caryophyllaceae, Caprifoliaceae, Convolvulaceae, Eleagnaceae, Fabaceae, Hypericaceae, Lamiaceae, Malvaceae, Onagraceae, Plumbaginaceae, Rosaceae, Scrophulariaceae. Pollen grains of Convolvulus sp. (33%) and Pyrus sp. (24%) were counted secondary. The pollen grains of 4 taxa belonging to Fabaceae, Lamiaceae and Plumbaginaceae family were determined as minor.

The results of the current study showed that Convolvulaceae, Rosaceae, Fabaceae, Asteraceae, Lamiaceae are the most preferred families by the honey bees. Behçet and Yapar (2019) has also reported that the top 5 families, which are Asteraceae, Lamiaceae, Fabaceae, Rosaceae, Apiaceae, are important for beekeeping in Bingöl Matan Mountains. Wróblewska *et al.* (2006) conducted a pollen analysis in 10 bee bread samples from North East Poland. They found the pollens of the genus Anthriscus, Brassicaceae, Centaurea cyanus and Trifolium repens in the highest frequency. In current study, dominant plant taxa were not found, but similar to this study, the frequency of Convolvulus pollen was found in range of 25-50% (Wróblewska *et al.*, 2006). In the Brazilian bee bread samples, 32 pollen types belonging to 27 genus and 22 families were identified. As a result of microscopic analysis, the most common pollen types belonged to Asteraceae, Mimosaceae, Euphorbiaceae, Lythraceae, Moraceae, Poaceae, Rubiaceae, Sapindaceae, and Tiliaceae families (Luz & Barth, 2012). Kaplan *et al.* (2019) conducted a study on five bee bread samples from Asteraceae, Fabaceae and Brassicaceae families at minor and rare levels. Mayda *et al.* (2020) found that Asteraceae, Fabaceae, Plantaginaceae and Rosaceae were common families in bee bread samples from Türkiye.

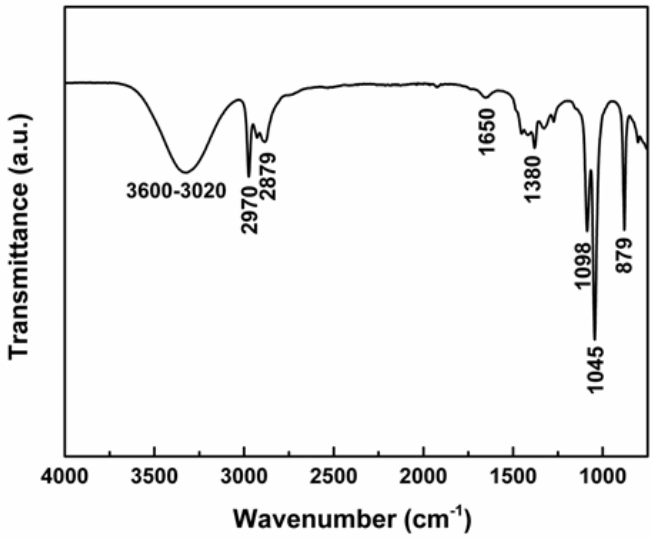
In our study Asteraceae, Fabaceae, Convolvulaceae and Rosaceae families were the most common.

Table 1. Frequency of pollen taxa in bee bread.

%	Pollen taxa
45-100 dominant	none
16-44 secondary	*Convolvulus sp., Pyrus sp.,*
3-15 minor	Lamiaceae, *Astragalus sp., Trifolium sp., Acantholimon Type I.*
<3 sporadic	Asteraceae, *Cichorium sp.,* Caryophyllaceae, Fabaceae, *Astragalus gummifer, Verbascum sp., Hedysarum sp., Lotus sp., Melilotus sp., Trifolium pratense, Eleagnus sp., Lamium sp., Malvaceae, Epilobium sp., Acantholimon TypeII,* Rosaceae, *Fragaria sp., Scabiosa sp., Hypericum sp.,*

The FTIR graph of bee bread extraction is given in Figure 2. According to the FTIR results, the wavenumber had the peak values of 3600-3020 cm^{-1} had the properties of O-H stretching vibration (Kuptsov & Zhizhin, 1998). 2970 cm^{-1} and 2879 cm^{-1} showed the properties of CH_3 and CH_2 asymmetric stretching vibration, respectively (Kostova, 2006). Carboxylate anion (COO-) was seen at the peak value of 1650 cm^{-1} (Kuptsov & Zhizhin, 1998). O-H bending in –COOH or/and CH_3 bending was/were seen at 1380 cm^{-1} (Kostova, 2006). Asymmetric vibration of ester link (C-O-C), C-O stretch in CH_3-COO-R, and predominantly C-C stretch corresponded to the peak value of 1098, 1045, and 879 cm^{-1}, respectively (Kostova, 2006; Kuptsov & Zhizhin, 1998).

Figure 2. FTIR graph as wave number versus transmittance of bee bread extract.



GC-MS graph of the EBB was given in Figure 3 and compound names, properties and quantities obtained from GC-MS results were given in Table 2. Figure 3 and Table 2 showed that compounds of different properties determined in the EBB. According to the Table 2, the compounds identified as Hexadecanoic acid, Hexadecanoic acid ethyl ester, Octadecanoic acid, Octadecanoic acid ethyl ester, 9,12,15-Octadecatrienoic acid methyl ester (Z,Z,Z), Methyl-3-(3,5-Ditertbutyl-4-Hydroxyphenyl) Propionate, Cholest-5-En-3-Ol(3Beta), Ruthenium organometallic ($C_{14}H_{21}BO_3RuSeSi$), and Bornyl ester of 3-isopropylidene cyclopentane carboxylic acid were seen over area of 1%. The structure types as fatty acids, cyclic, aromatic, phenolic, terpenoid, diterpen and metallic complex structures were seen in GC-MS results.

Figure 3. GC-MS graph as time versus abundance of bee bread extract.

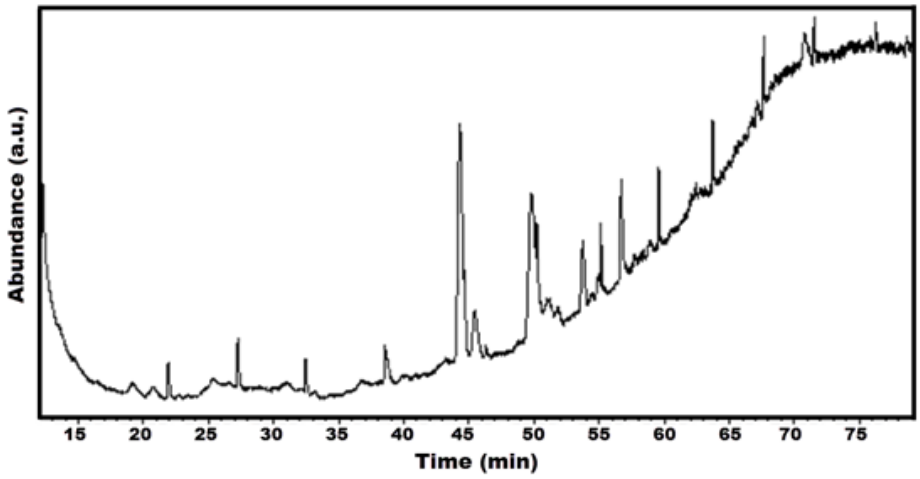


Table 2. The data about compound names, properties and quantities obtained from GC-MS results.

RT	Area%	Library/ID	Structure Type	Ref#	CAS#	Qual
44.290	9.13	Palmitic acid (Hexadecanoic acid)	Fatty acid	12	000112-39-0	99
45.469	2.19	Hexadecanoic acid, ethyl ester	Fatty acid	696	000628-97-7	89
49.777	6.30	Stearic acid (Octadecanoic acid)	Fatty acid	16	000112-61-8	97
51.168	1.75	Octadecanoic acid, ethyl ester	Fatty acid	151552	000111-61-5	94
51.803	0.65	Methyl linoleate	Fatty acid	133505	000112-63-0	90
53.743	1.87	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	Fatty acid	22	000301-00-8	96
54.944	0.50	4-Pentyl-1-(4-Propylcyclohexyl)-1-Cyclohexene	Cyclic	133389	108067-17-0	92
55.139	0.75	Benzo[G]Quinoline-2-Carboxylic-Acid	Aromatic	522489	071295-05-1	80
56.684	2.21	Methyl-3-(3,5-Ditertbutyl-4-Hydroxyphenyl)-Propionate	Phenolic	469837	000000-00-0	95
62.692	0.95	4-(4-Ethylcyclohexyl)-1-Pentyl-1-Cyclohexene	Cyclic	59858	301643-32-3	90
63.121	0.63	1,5-Dimethyl-2-Vinylcyclohexanecarboxylic-Acid	Cyclic	286470	106542-17-0	86
64.203	0.20	1-Allyl-3-Methylindole-2-Carbaldehyde	Terpenoid	55113	000000-00-0	90
65.456	1.52	Cholest-5-En-3-Ol(3.Beta.) /Cholesterol	Terpenoid	57079	000057-88-5	90
70.411	0.58	1,5-Dimethyl-2-Vinylcyclohexanecarboxylic-Acid	Cyclic	286470	106542-17-0	83
71.504	3.12	Ruthenium organometallic ($C_{14}H_{21}BO_3RuSeSi$)	Metallic Complex	2028	118772-38-6	90
73.552	3.03	Ruthenium organometallic ($C_{14}H_{21}BO_3RuSeSi$)	Metallic Complex	2028	118772-38-6	92
73.650	0.32	(7R,14S,1E,3E,8E,11E)-Cembra-1,3,8,11-Tetraene-7,14-Diol	Diterpen	287197	000000-00-0	83
75.023	1.21	Bornyl-Ester-Of-3-Isopropylidene-Cyclopentanecarboxylic-Acid	Cyclic	287113	000000-00-0	86

FTIR results showed properties of O-H stretching, CH_3 and CH_2 stretching vibration, carboxylate anion peak, O-H bending in –COOH, CH_3 bending, ester link (C-O-C), C-O stretch in CH_3-COO-R, and predominantly C-C stretching according to literature data. C-C, CH_2 and CH_3 structure types, which have the longest peak value, were generally seen in all of the compounds. Especially, -COOH structure types were seen in fatty acids and carboxylic acid compounds. C-O-C and CH3-COO-R structure types were also seen in the compounds with ester; the compounds of bornyl ester of 3-isopropylidene cyclopentane carboxylic acid, Methyl-3-(3,5-Ditertbutyl-4-Hydroxyphenyl) Propionate and (7R, 14S, 1E, 3E, 8E,1 1E)-Cembra-1,3,8,11-Tetraene-7,14-Diol. As it was seen in Figure 2, FTIR results were compatible with GC-MS results and the structure types of FTIR results were seen in the dominant compounds of GC-MS results. The GC-MS results showed EBB had different properties such as antibacterial, antioxidant, anticancer activities etc., when the structures were based on the GC-MS results. In the literature data, complex structures containing ruthenium exhibited anticancer activity due to rapid ligand exchange, biological stability, and different and high oxidation states thanks to ruthenium (Reedijk, 2008; Syamdidi & Irianto, 2016). Cholest-5-En-3-Ol (3Beta) compound had antioxidant activity (Greenland & Bowden, 1994; Khan *et al.*, 2019). (7R, 14S, 1E, 3E, 8E,1 1E)-Cembra-1,3,8,11-Tetraene-7,14-Diol molecule named as cytotoxic diterpene was detected showing antitumor effect (Chandrasekaran *et al.*, 2008). Fatty acids, their methyl and ethyl ester compounds showed antibacterial, antioxidant and anticancer activities (Akbari *et al.*, 2019; Ghosh & Indra, 2014; Pinto *et al.*, 2017). It was seen that the structures with bornyl ester, cyclopentanecarboxylic acid and cyclohexanecarboxylic acid showed anti-imflammatory and antimicrobial activities (Bakour *et al.*, 2019; Etehadpour & Tavassolian, 2019; Soumya *et al.*, 2014). Bee bread can be used as a healthy food and a supplement for medicine (Magalhães *et al.*, 2008; Mărgăoan *et al.*, 2019). Bee bread has a unique chemical composition providing antioxidant, antimicrobial and cytotoxic effects (Magalhães *et al.*, 2008; Mărgăoan *et al.*, 2019; Sobral *et al.*, 2017).

There are several methods to evaluate the antioxidant activity of bee bread in the literature (Sreeramulu *et al.*, 2013). DPPH free radical-scavenging activity was chosen for this study to evaluate the antioxidant property at different concentrations of EBB. DPPH radical scavenging activities of bee bread and its EC50 values are given in Table 3. The scavenging activity (RSA) of EBB showed inhibition variability between 20.15 ± 0.68% and 93.18 ± 0.44% depending on the concentration. The 200 mg/mL sample presented the best RSA, while sample 25 mg/mL showed the worst performance. In addition, the EC50 value of bee bread sample was measured as 80.08 ± 0.10 mg/mL.

Table 3. DPPH radical scavenging activities (RSA) and EC50 value of bee bread extracts at different concentrations.

EBB	Concentrations				EC50 (mg/mL)
	25 mg/mL	50 mg/mL	100 mg/mL	200 mg/mL	
RSA(%)	20.15 ± 0.68	48.20 ± 0.44	76.93 ± 0.34	93.18 ± 0.44	80.08 ± 0.10

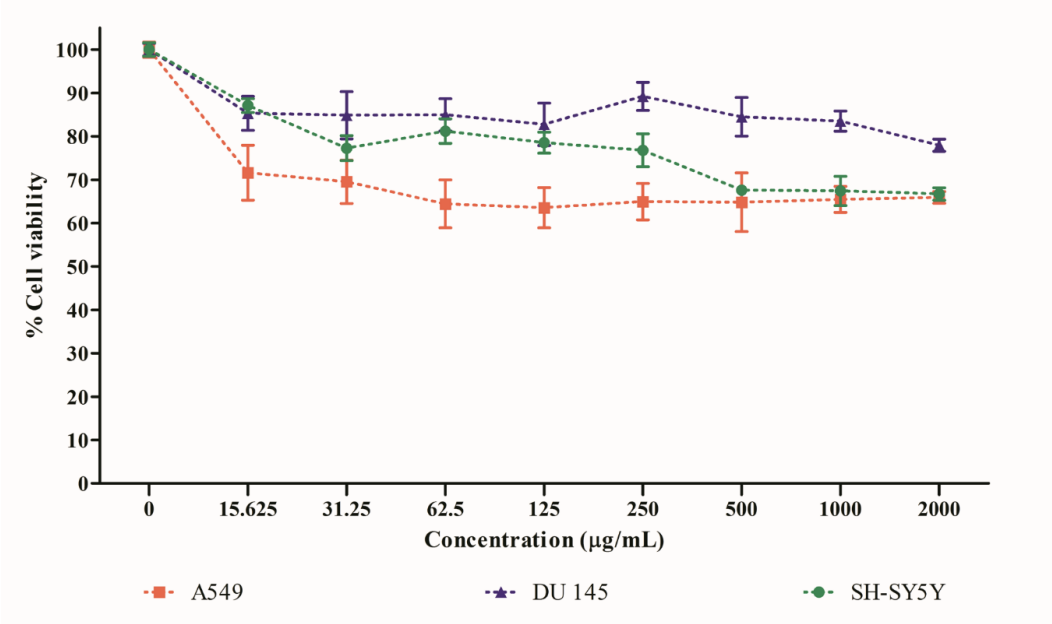
All data were expressed as mean ± SD of triplicate.

All values ranked it among the powerful antioxidant foods (Li *et al.*, 2014). This high antioxidant property of EBB can be probably due to the functional compounds in its content such as phenolic compound (Methyl-3-(3,5-Ditertbutyl-4-Hydroxyphenyl)-Propionate), aromatic group (Benzo[G]Quinoline-2-Carboxylic-Acid), and terpenoid (Cholest-5-En-3-Ol(3Beta)) (Borawska *et al.*, 2014; Greenland & Bowden, 1994; Khan *et al.*, 2019).

Bee bread has also cytotoxic activity on tumor cells besides antioxidant effects but there are a few researches on cytotoxic effect on tumor cells of bee bread in the literature. In this study,

we examined the cytotoxic effects of EBB in concentrations of 15.625-2000 µg/mL on human lung adenocarcinoma (A549), human prostate cancer (DU 145), and human neuroblastoma (SH-SY5Y) cell lines and we found that EBB exhibited moderately cytotoxic effect on all of 3 tumor cell lines (A549, DU 145, and SH-SY5H) at all concentrations with 24h incubation and decreased cell viabilities to 63.55, 77.94, and 66.71% respectively. Figure 4 indicates the results of cell viability expressed as a percentage of the control after a 24-hour incubation using an EBB concentration range of 15.625 - 2000 µg/mL.

Figure 4. Viabilities of A549, DU 145, and SH-SY5Y cancer cells (% of the control) after incubation with bee bread extract. Values are presented as mean ± SEM and are statistically significant at $p<0.05$.



EBB exhibited moderate cytotoxic activity at all concentrations against A549, DU 145 and SH-SY5Y cancer cells. It showed the maximum cytotoxic effect at 125 µg/mL concentration with 63.55 ± 8.03% viability on A549 cell line, at 2000 µg/mL concentration with 77.94 ± 2.41% viability on DU 145 cell line, and at 2000 µg/mL concentration with 66.71 ± 2.48% viability on SH-SY5Y cell line. These values were statistically significant compared to the control. Markiewicz-Żukowska *et al.* (2013) studied cytotoxic effect of 3 different ethanolic EBBs in concentrations of 10–100 µg/mL on U87MG cell lines and they reported that, after incubation of 24 h, while one of the 3 different EBBs showed a moderately cytotoxic effect, the other two did not show any cytotoxic effect. Borawska *et al.* (2014) examined the effects of EBB in concentration of 50 µg/mL on astrocytoma (DASC), human glioblastoma multiforme (U87MG), and normal human astroglia (SVGp12) cell lines. They demonstrated that, in a 24 h incubation, while EBB moderately inhibits the growth of U87MG and SVGp12 cells, but not DASC. Sobral *et al.* (2017) studied the effects of bee bread against some human tumor cells. Bee bread extracts collected from northeastern Portugal were tested on breast adenocarcinoma, hepatocellular carcinoma, cervical carcinoma and non-tumor liver cells, and also against non-small cell lung cancer. The extracted bee bread showed normal levels of antitumor activity; but, extractions did not cause toxicity in normal cells (Sobral *et al.*, 2017). The other studies conducted on different cell lines confirmed the antitumor activity of bee bread.

Based on all of this data, it can be said that the EBB had moderately cytotoxic effects on cancer cells, and this data matched up with our cell viability test. Cytotoxic property of EBB can be probably due to the presence of another functional compound in its content such as Ruthenium organometallic ($C_{14}H_{21}BO_3RuSeSi$) groups, (7R,14S,1E,3E,8E,11E)-Cembra-

1,3,8,11-Tetraene-7,14-Diol molecule named as cytotoxic diterpene, fatty acids, fatty acid methyl esters, and fatty acid ethyl ester compounds (Akbari *et al.*, 2019; Chandrasekaran *et al.*, 2008; Ghosh & Indra, 2014; Pinto *et al.*, 2017; Reedijk, 2008; Syamdidi & Irianto, 2016). In addition, more studies are needed to clarify the anticancer mechanisms of bee bread.

4. CONCLUSION

The composition of bee bread varies depending on the botanical origin. There are endemic plants in Bingöl-Türkiye. Bee bread is less studied than pollen and the existence of a region-specific flora reveals the importance of the studies in this region. Bee bread is anti-cancer, protects the nervous system and is effective against viruses. Cancer has many treatment methods; despite therapeutic and diagnostic drugs, it is one of the deadliest diseases in the world. The main reason for this is the damage of the immune system in the treatment of tumors. Therefore, there is a need to reveal new anti-tumor molecules that strengthen the immune system without harming the person. The ingredients in bee bread support the immune system. Also, bee bread has cytotoxic activity on tumor cells beside of antioxidant and antimicrobial effects but there are a few researches on cytotoxic effect on tumor cells of bee bread in the literature. In our study EBB exhibited moderate cytotoxic activity at all concentrations against A549, DU 145, and SH-SY5Y cancer cells. We think that our study will give an idea about cancer research and drug development studies.

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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

Gokhan Dervisoglu: Performed the experimental part and statistical analysis, wrote and edited the original draft. **Duygu Nur Cobanoglu** and **Serhat Kocyigit**: did the experimental part, helped write the draft. **Sedat Yelkovan** and **Davut Karahan**: helped to carry out the experimental part and to write the draft. **Yusuf Cakir**: helped carry out the experimental part.

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Research Article

Effect of culture time on secondary metabolite production from MeJA-stimulated *in vitro* roots of *Hyoscyamus niger* L.

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Abstract: This study was conducted to determine the effects of different culture times on root growth and accumulation of secondary metabolites in adventitious roots from *Hyoscyamus niger* petioles. For this purpose, adventitious roots transferred to culture media containing 1 mM methyl jasmonate (MeJA) were harvested at 11 different culture times, starting from day 1 to day 30. After harvest, the fresh and dry weights of the roots and the root growth index as parameters of root growth, as well as the amounts of tropane alkaloids and phenolic compounds, were examined. It was found that the fresh and dry weights and the growth index of the roots harvested between the 12th and 30th day were the highest. The highest amounts of scopolamine were obtained from roots harvested on days 15, 18, and 21. The highest hyoscyamine accumulation was found on days 12 and 15. The highest total phenolic content was 19.33 mg g^{-1} in root cultures harvested on day 15. HPLC analyses revealed that ferulic acid, caffeic acid, *o*-coumaric acid, *p*-coumaric acid, rutin, and quercetin were not detected in the roots. On the other hand, the amounts of gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid, and chlorogenic acid showed significant changes depending on the culture times. Considering all the results, it was found that the optimum culture time for the production of secondary metabolites in *H. niger* adventitious root cultures stimulated with 1mM MeJA was 15 days.

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1. INTRODUCTION

Tropane alkaloids (TAs), important secondary metabolites (SMs) with various pharmaceutical effects (Kohnen-Johannsen & Kayser 2019), occur in several families such as Proteaceae, Convolvulaceae, Brassicaceae, Euphorbiaceae, Rhizophoraceae, Solanaceae, and Erythroxylaceae (Jirschitzka *et al.,* 2012). In particular, plants of the Solanaceae family have been used as medicine for centuries due to their richness in TAs. *Hyoscyamus niger*, an annual or biennial herbaceous plant belonging to Solanaceae, is one of the most important species for the pharmaceutical industry due to its high content of TAs such as hyoscyamine and scopolamine, which are among the oldest drugs used in medicine (Dehghan *et al.,* 2012;

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Pudersell *et al.,* 2012). Hyoscyamine and scopolamine are generally used to treat stomach aches, earaches, sinusitis, ulcers, kidney, and liver ailments, as well as to relieve pain and as antispasmodics (John *et al.,* 2010; Sargın *et al.,* 2013). *H. niger* acts on the parasympathetic nervous system due to its TAs and is used to treat diseases of the ocular, anaesthetic, cardiac, and digestive systems (John *et al.,* 2010). Scopolamine has been found to have beneficial effects in patients with acute vertigo and to reduce gastric acid secretion during sleep in patients with nausea and ulcers caused by chemotherapy drugs in cancer patients (Clissold & Heel 1985). Scopolamine is a valuable pharmaceutical raw material because it has higher pharmacological activity and fewer side effects than hyoscyamine. Therefore, it is now known that the demand for scopolamine is about 10 times higher than that for hyoscyamine (Qin *et al.,* 2014; Shakeran *et al.,* 2017). Apart from these important medicinal effects, hyoscyamine and scopolamine can be highly toxic to the human body if consumed unknowingly. When the roots and leaves of *H. niger* are consumed as food, scopolamine and hyoscyamine can cause intoxication by paralysing the nerve endings of the parasympathetic system (Orbak *et al.,* 1998). *H. niger* poisoning has many clinical symptoms such as mydriasis, tachycardia, arrhythmia, agitation, convulsive thirst, dry mouth, nausea, vomiting, headache, blurred vision, auditory, visual, or tactile hallucinations, disorientation, and aggression (Alizadeh *et al.,* 2014).

H. niger is native to Europe and North Africa (Pokorny *et al.,* 2010). However, it is widely distributed in regions with temperate climates (Alizadeh *et al.,* 2014). It grows naturally in rocky areas, on undeveloped land, and on roadsides (Li *et al.,* 2011; Yücel & Yılmaz, 2014). An erect, viscid-haired, greasy, annual or biennial herb, 30-150 cm tall. Leaves are radical and cauline, neatly toothed or pinnately lobed. Flowers are bisexual, regular, pale yellowish-green or yellowish-white with a dark purple (reticulate) center, stalkless or subsessile, in axillary or terminal scorpion-like umbels. Capsules are surrounded by the globose base of the enlarged calyx. Seeds are oval, kidney- or knee-shaped, about 1.5 mm in diameter, brown, with fine but conspicuous reticulations. Flowering and fruiting occur from April to September.

The use of plants as medicines in traditional Chinese medicine dates back to 2700 BC, and the medicinal effects of many plants were discovered through trial and error (Faydaoğlu & Sürücüoğlu, 2013). Nowadays, medicinal plants, which occupy an important place in drug discovery studies, continue to be a natural source of pharmaceutical raw materials due to their content of SMs (D'yakova *et al.,* 2020; Zvezdina *et al.,* 2020). As the consumption of herbal medicines continues to increase, the reliance on medicinal plants to obtain active pharmaceutical ingredients has become even greater (Hong *et al.,* 2012). Synthetic production of pharmaceutical compounds, especially alkaloids found in plants, is more expensive and difficult than their isolation from plants due to the complexity of their chemical structures and the length of biosynthetic pathways (Dehghan *et al.,* 2012). For this reason, plant tissue cultures are now commonly used to achieve higher alkaloid production (Shah *et al.,* 2020). The production of SMs with medicinally important effects by *in vitro* methods should be provided in stable and desired quantities, regardless of seasonal variations compared to wild-collected or field-grown plants. At the same time, the use of these methods can prevent the uncontrolled collection of plants from their natural habitats (Ajungla *et al.,* 2009; Shah *et al.,* 2020; Roy 2021). *İn vitro* root cultures are one of the most successful methods for obtaining large amounts of valuable SMs from the roots of many plants (Kareem *et al.,* 2019; Roy 2021). However, low productivity often proves to be a major problem in the *in vitro* production of secondary metabolites (SMPs). To overcome this problem, some applications are made, such as changes in nutrient content, culture conditions, or the addition of new chemicals (Lu *et al.,* 2020). The use of biotic and abiotic elicitors *in vitro* root cultures is considered one of the most acceptable strategies to increase the production of many SMs such as phytoalexins and alkaloids in roots (Shah *et al.,* 2020). Various biotic and abiotic elicitors added to the culture medium can increase the production of SMs by activating the appropriate genes for synthesis or stimulating

physiological processes that lead to further accumulation of SMs (Shah *et al.,* 2020). Jasmonic acid and its methyl ester methyl jasmonate (MeJA) play a role in various physiological developmental processes such as seed germination, root growth, fertility, fruit ripening, and activation of the plant defense system against biotic and abiotic stresses (Zhang *et al.,* 2020; Zuniga *et al.,* 2020; Wang *et al.,* 2021). MeJA, first identified as a component of the essential oils of *Jasminum grandiforum* L. and *Rosmarinus officinalis* L., assist in the biosynthesis of various SMs such as terpenoids, indole alkaloids, nicotine, flavonoids, ginsenosides, benzophenanthridine alkaloids, and glucosinolates (Giri & Zaheer 2016; Tang *et al.,* 2020; Yousefian *et al.,* 2020). MeJA increases the amount of important TAs such as hyoscyamine and scopolamine by directly or indirectly affecting the synthesis of key enzymes such as Putrescine-N-methyl transferase (PMT) and hyoscyamine-6 beta-hydroxylase (H6H), which are responsible for the biosynthesis of these alkaloids (Kang *et al.,* 2004).

Culture time is another important factor determining yield in the *in vitro* production of plant SMs (Demirci *et al.,* 2020; Demirci *et al.,* 2021; Demirci *et al.,* 2022; Narayani and Srivastava 2017). In nature, each SM reaches its highest level at different time periods (Figueiredo *et al.,* 2008; Soni *et al.,* 2015). In *in vitro* cultures, the SM levels also change at different culture times, and it is important to determine the optimal culture time for each metabolite.

To our knowledge, there are no studies investigating the effects of different culture times on root growth, synthesis of TAs, and accumulation of phenolic compounds in MeJA-stimulated *H. niger* adventitious root cultures. The objective of this study was to find the optimal culture time in terms of root quantity, TAs, and phenolic compounds to achieve maximum yield from *H. niger* adventitious root cultures. This study provides important insights for future scale-up research with bioreactors.

2. MATERIAL and METHODS

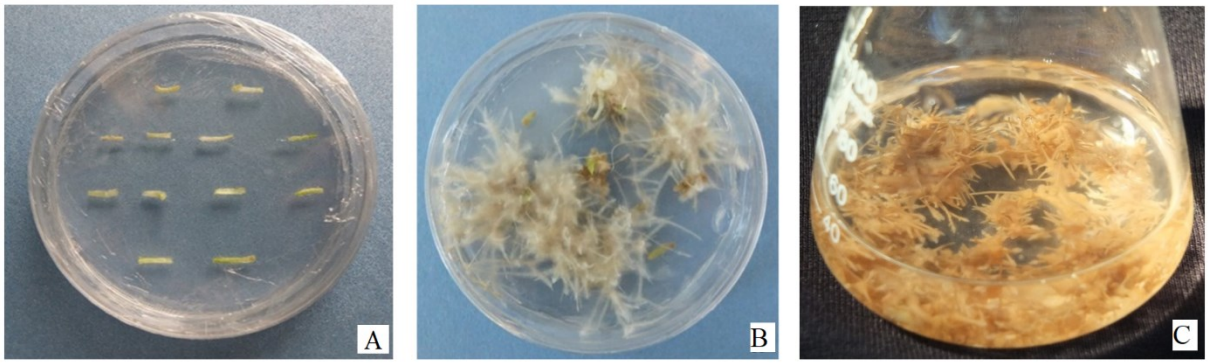
2.1. Plant Material

H. niger seeds used in this study were obtained from the Garden Directorate of Medicinal and Aromatic Plants of Zeytinburnu Municipality, Turkey. After the seeds germinated under *in vitro* conditions, petiole explants taken from the plantlets were used to form adventitious roots. These adventitious roots were then used as plant material in this study.

2.2. Obtaining Adventitious Roots

Seed germination and adventitious root collection followed the methods described in detail in our previous study (Demirci *et al.,* 2022). Briefly, seeds treated with gibberellic acid for 48 hours were rinsed with double distilled water (ddH_2O) and then sterilized by shaking with 70% ethanol (%99, Tekkim, Turkey) for 10 seconds and with 0.1% mercury chloride ($HgCl_2$, Sigma-Aldrich, Germany) for 10 min. The sterile seeds rinsed with sterile ddH_2O were transferred to Murashige and Skoog (1962) media (Duchefa Biochemie, Netherland) containing 3% sucrose (Sigma-Aldrich, Germany) and 0.6% agar (Sigma-Aldrich, Germany) and cultured in the dark at 25°C for 15 days. Germinated seeds were transferred to the media described above in tissue culture boxes with a volume of 250 ml and cultured for 15 days at 25°C (16 hours light/8 hours dark). Petiole explants of 1 cm length obtained from plantlets in growth media were cultured in MS media containing 2 mg l^{-1} indole-3-butyric acid (IBA, Sigma-Aldrich, Germany), 3% sucrose, and 0.6% agar for 6 weeks at 25°C in the dark to form adventitious roots (Figure 1-A). The adventitious roots formed (Figure 1-B) were transferred to 30 ml of liquid MS (2 mg l^{-1} IBA and 3% sucrose) medium in 100 ml culture boxes and cultured at 25°C (Figure 1-C). Roots growing on liquid media were subcultured onto the same liquid media at 3-week intervals.

Figure 1. Obtaining of *in vitro* adventitious roots in *Hyoscyamus niger* (A: inoculation of petiole explant, B: formation of adventitious roots, C: propagation of adventitious roots in liquid medium).



2.3. Stimulation of Adventitious Roots with MeJA

Healthy growing adventitious roots in liquid MS medium were weighed 1.25 g under aseptic conditions using an analytical balance and transferred to liquid MS medium supplemented with 2 mg l^{-1} IBA and 30 g l^{-1} sucrose. After the roots were cultured in these culture media at 25 °C for 7 days, 1 mM MeJA (Sigma-Aldrich, Germany) was added to the culture media. The stock solution of MeJA prepared with pure ethanol was added to each flask at 100 µl after filter sterilization. Roots were harvested on days 1, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 to study the effects of culture times after the application of 1mM MeJA. MeJA applications were made in 3 replicates for each harvest time and 4 flasks per replicate (11 harvest times X 3 replicates X 4 flasks). All applications and analyzes were planned as a completely randomized design.

2.4. Determination of Root Growth Parameters

Adventitious roots harvested at 11 different culture times were washed and the culture medium on the roots was completely removed. The fresh weights of the roots dehydrated with blotting paper were first determined on the analytical balance and calculated as g 100 ml^{-1}. The following formula was used to calculate the growth index of the roots whose fresh weights were determined: Growth index: (harvested FW (g) - inoculated FW (g)) / inoculated FW (g).

After the roots were completely dried in a drying oven at 40°C for 72 hours, they were weighed and the dry weights of the roots were calculated in g 100 ml^{-1}.

2.5. Extraction of Tropan Alkaloids and Phenolic Compounds from Adventitious Roots

To analyses the TAs and phenolics by chromatographic methods, the dried roots were ground with a pestle until they were powdered. Two hundred mg of the powdered roots were weighed and mixed with 20 ml of methanol solution (HPLC grade, Sigma Aldrich, Germany) (methanol:ddH2O, 60:40 (v/v)) in 50 ml test tubes. The mixture was shaken in an ultrasonic water bath for 15 min and centrifuged at 9000 rpm for 15 min. The same procedure was repeated two more times for the pellet fraction, while the supernatant fractions were transferred to flasks. Then, the collected supernatants were evaporated with a rotary evaporator at 45 °C under a vacuum to obtain dry extracts. To the dry extract, 1.5 ml of methanol was added and completely dissolved by vortexing. The extracts were stored at -20 °C after being filtered at 0.45 µm for analysis by HPLC and spectrophotometer (Jakabova *et al.,* 2012).

2.6. Determination of Tropane Alkaloids by HPLC

Chromatographic analyses of TAs were performed using a Shimadzu HPLC system (Kyoto, Japan). The HPLC system consisted of a LC -20AD pump, a DGU-20A3R degasser, a CTO -10AS VP column heater, an SPD-M20A diode array detector, and a 250 × 4.6 mm i.d. 5 µm HPLC column (Agilent Eclipse XDB-C18, Wellborn, Germany). The modified method of

Boitel-Conti *et al.,* (2000) was used to determine the amount of hyoscyamine and scopolamine by HPLC. Mobile phase A was 2% acedic acid (Sigma-Aldrich, Germany) ultrapure water, and mobile phase B was 100% acetonitrile (HPLC grade, Sigma-Aldrich). Separation was performed according to the gradient programme; 0-12% B, 0-12 min; 12-20% B, 12-13 min; 20-28% B, 13-33 min; 28-100% B, 33-48 min. Flow rate and column temperature were 0.8 ml min^{-1} and 40 °C, respectively. The injection volume was 20 µl. Calculations were made at 220 nm in the instrument software (Shimadzu Class- VP Chromatography Laboratory Automated Software system) according to the calibration prepared with the analytical standards scopolamine (Sigma-Aldrich, Germany) and hyoscyamine (Sigma-Aldrich, Germany) and were expressed as mg g^{-1} dry weight. The data are the average of three measurements.

2.7. Determination of Phenolic Compounds by HPLC

The amounts of phenolic compounds including gallic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, vanillin, rosmarinic acid, *p*-coumaric acid, *o*-coumaric acid, ferulic acid, rutin, cinnamic acid, and quercetin in adventitious roots were determined by HPLC. The HPLC system and column were the same as those used for the determination of TAs. For gradient elution, mobile phase A contained ultrapure water with 2% acetic acid, and mobile phase B contained 100% methanol (HPLC grade, Sigma-Aldrich). The HPLC gradient, 0-12% B, 0-12 min; 12-20% B, 12-13 min; 20-28% B, 13-33 min; 28-30% B, 33-48 min; 30-38% B, 48-53 min; 38-40% B, 53-68 min; 40% B ,68-70 min; 40-50% B ,70-90 min; 50-60% B ,90-105 min; 60-100% B ,105-107 min; 100% B ,107-112 min; 0% B ,112-117 min. Flow rate and column temperature were set at 0.8 ml min-1 and 40 °C. The injection volume was 20 µl. Calculations were performed in the instrument software (Shimadzu Class- VP Chromatography Laboratory Automated Software system) according to the calibration established with analytical standards and expressed as µg g^{-1} dry weight. The data are an average of three measurements.

2.8. Determination of Total Phenolic Content (TPC)

Total phenolics of adventitious roots were determined by the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). Accordingly, 40 µl of the liquid extracts obtained in the extraction phase were transferred to test tubes. Then, 2.4 ml of distilled water and 200 µl of Folin Ciocalteu were added to each. After waiting for 5 min, 600 µl of a saturated sodium carbonate solution at room temperature and 760 µl of distilled water were added to the mixtures. After vortexing, the mixture was incubated for 2 h at room temperature in the dark. Absorbances were measured at 765 nm using a spectrophotometer (T70 Plus Dual Beam/Arlington, USA). The TPC in the roots was calculated using the calibration curve of gallic acid standards and the results were expressed as mg gallic acid equivalents (GAE) g^{-1} DW).

2.9. Statistical Analyses

The root cultures were randomly selected during MeJA applications and roots in each culture medium in which the application occurred were examined in the experiments. Data were analyzed at the $p \leq 0.05$ level using Duncan' Multiple Range Test with the program IBM SPSS 22 (ANOVA).

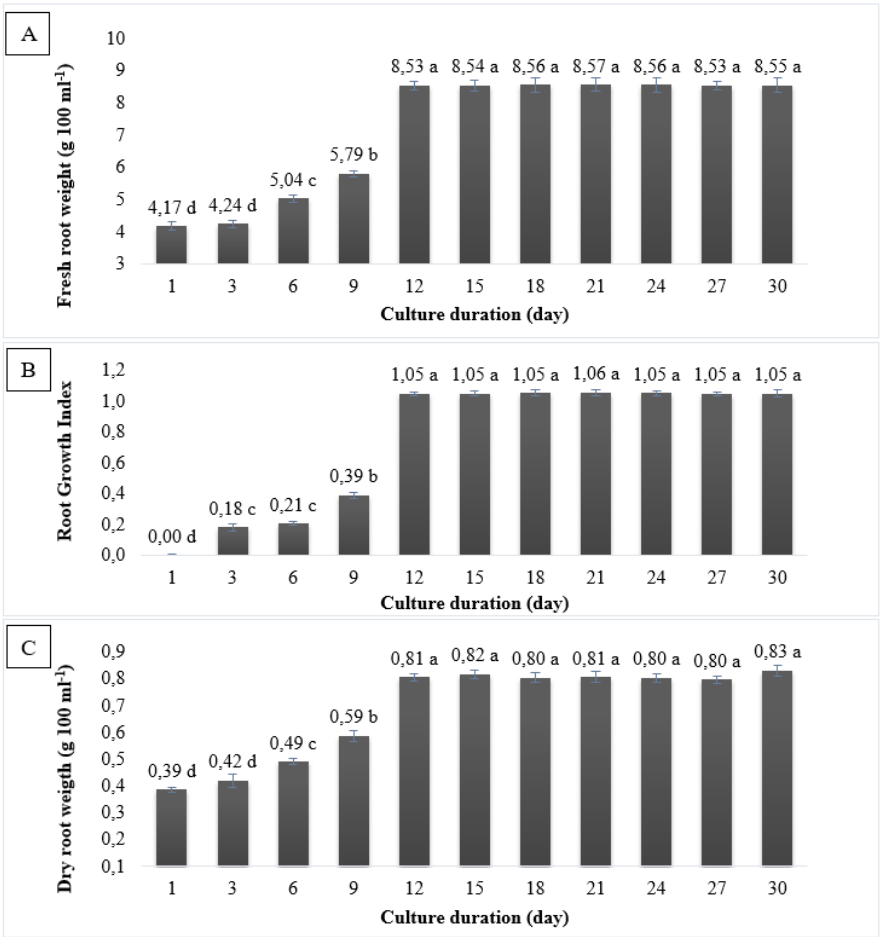
3. RESULTS

3.1. Effects of Culture Times on The Parameters of Root Growth

Root fresh weights, root growth indexes, and root dry weights were studied as root growth parameters. There were statistically significant differences in all growth parameters depending on the culture times ($p \leq 0.05$). The lowest fresh weights were found in roots harvested on the 1st and 3rd day after the application of 1 mM MeJA (4.17-4.24 g 100 ml^{-1}). Fresh weights increased significantly on days 6, 9, and 12 of culture with values of 5.04, 5.79, and 8.53 g 100 ml^{-1}, respectively (Figure 2A). However, no significant differences were observed between

harvests grown at 3-day intervals from day 12 to day 30. The root growth index increased slowly from day 3 and peaked (1.05) on day 12 (Figure 2B). Thereafter, the growth index remained constant until day 30 and showed no significant differences. Root dry weights were lowest on days 1 and 3 and increased significantly on days 6, 9, and 12, similar to fresh weight and growth index. While root dry weights ranged from 0.80 to 0.82 at all culture times, there were no significant differences between the 12th and 30th days. The lowest root dry weights ranged from 0.39 to 0.42 g 100 ml^{-1} and the highest root dry weights ranged from 0.80 to 0.83 g 100 ml^{-1}. Based on these results, it was determined that the optimum values in terms of root growth parameters were obtained for roots cultured during 12, 15, 18, 21, 24, 27, and 30 days.

Figure 2. Effects of culture times on the root growth parameters in *Hyoscyamus niger* adventitious roots stimulated with 1 mM MeJA (A: fresh root weight (FW, g 100 ml^{-1}), B: root growth index (GI), C: dry root weight (DW, g 100 ml^{-1})) (Duncan's multiple range test, $p \leq 0.05$).

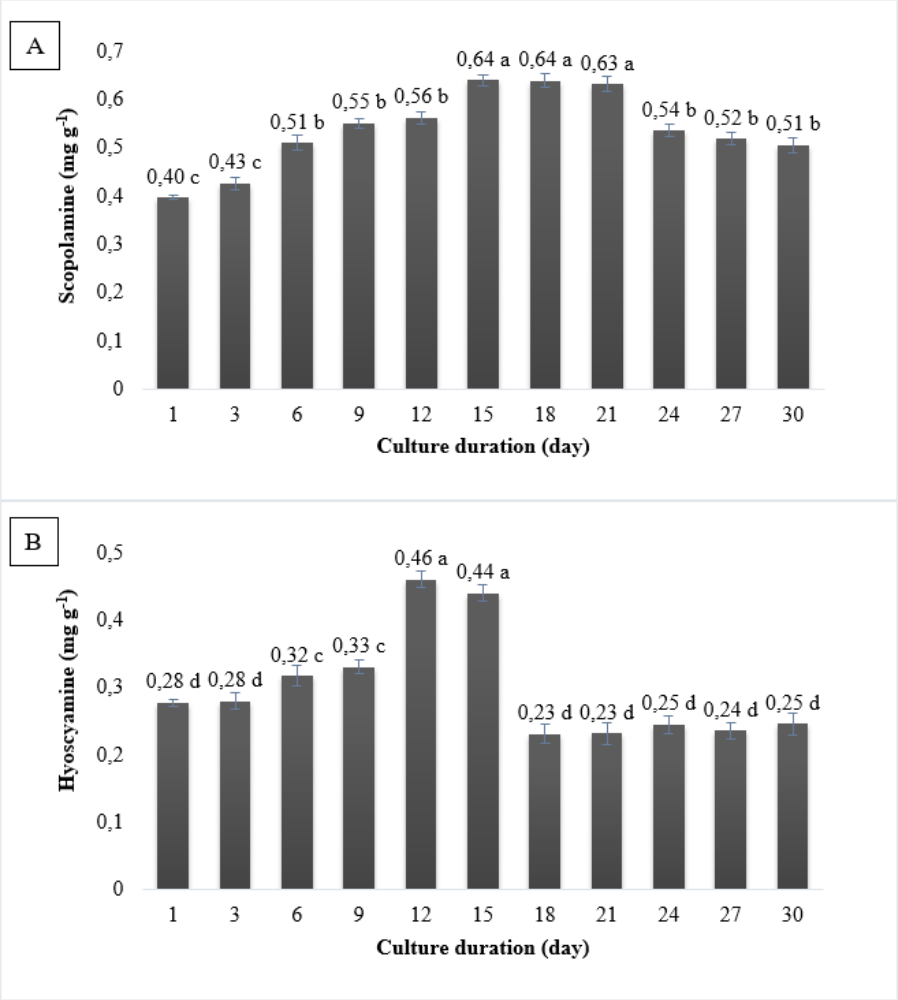


3.2. Effects of The Culture Times on The Production of Tropane Alkaloids

To understand the effects of culture times on the accumulation of tropane alkaloids in adventitious roots treated with 1 mM MeJA, the amount of hyoscyamine and scopolamine was determined by HPLC (Figure 3). The lowest amounts of scopolamine in the adventitious roots were detected at 0.40 and 0.43 mg g^{-1} on day 1 and day 3, respectively. The amount of scopolamine increased from day 3 to day 15, but there were no significant differences on days 6, 9, and 12 (0.51, 0.55, and 0.56 mg g^{-1}, respectively). The scopolamine level, which reached its maximum on day 15, remained constant between 0.63-0.64 mg g^{-1} on days 18 and 21, after which it decreased significantly on day 24 and remained constant until day 30 (Figure 3A). When the amount of hyoscyamine was analysed, it was 0.28 mg g^{-1} on days 1 and 3 and

increased to 0.32 and 0.33 mg g^{-1} on days 6 and 9, respectively. Then, it reached the highest values of 0.46 and 0.44 mg g^{-1} on days 12 and 15, respectively. However, the amount of hyoscyamine decreased from day 18 and remained between 0.23 mg g^{-1} and 0.25 mg g^{-1} until day 30 without significant change (Figure 3B). The analyses revealed that the 15-day culture period after the application of 1 mM MeJA was the optimum for the accumulation of hyoscyamine and scopolamine in adventitious root cultures of *H. niger*.

Figure 3. Effects of culture times on the scopolamine (A) and hyoscyamine (B) amounts in *H. niger* adventitious roots stimulated with 1 mM MeJA (Duncan's multiple range test, $p \leq 0.05$).



3.3. Effect of Culture Times on The Production of Phenolics

Total phenolic content (TPC) was determined by the spectrophotometric method to understand the effects of the culture times on the total phenolic accumulation in *H. niger* adventitious roots treated with 1 mM MeJA (Table 1). The TPC analyses revealed that the lowest TPCs were found in the roots harvested on day 1 at 5.59 mg g^{-1} and day 3 at 6.37 mg g^{-1}. The TPC increased from the 3rd day of culture and reached a maximum value of 19.33 mg g^{-1} on the 15th day. However, there was a significant decrease in the amount of TPC on the 18th day compared to the 15th day. It was found that there was no significant difference in TPC amount between the 18th and 21st day, while the decrease in TPC amount on the 24th day was not significant.

To determine the effects of culture times on the accumulation of some phenolic compounds, the amounts of gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid,

ferulic acid, caffeic acid, o-coumaric acid, p-coumaric acid, rutin, quercetin, and chlorogenic acid were also analysed by HPLC in this study. According to the results of HPLC analyses, caffeic acid, ferulic acid, rutin, quercetin, o-coumaric acid, and p-coumaric acid could not be detected in the roots. The amounts of gallic acid, catechin, epicatechin, vanillin, cinnamic acid, rosmarinic acid, and chlorogenic acid differed significantly according to culture time after the application of 1 mM MeJA ($p \leq 0.05$). Gallic acid levels reached their highest values on the 9th, 12th, and 15th days, with values ranging from 62.21 to 61.92 µg g^{-1}. From the 18th day, they began to decrease, and the lowest values were obtained in roots cultured for 27 and 30 days. Catechin showed no significant difference in the first 6 days of culture, but the increase that started on the 9th day reached the highest value on the 15th day. The lowest amount of catechin was found in the roots harvested on the 1st day with 97.49 µg g^{-1}, while the highest amounts of catechin were found in the roots collected on the 15th and 18th days with values of 598.77 and 596.10 µg g^{-1}, respectively. The accumulation of epicatechin in the roots reached its highest value of 28.02 µg g^{-1} on the 15th day. On day 18, the amount of epicatechin decreased drastically to 4.86 µg g^{-1}. While this drastic decrease in the amount of epicatechin continued until day 21, no epicatechin was detected on day 24 and the following days. HPLC analysis showed that the amount of vanillin increased rapidly from the first harvest period, reaching its highest value on day 6 and maintaining this value until day 15. However, on day 18 it began to decrease (14.15 µg g^{-1}) and reached its lowest value on day 21 (4.13 µg g^{-1}), decreasing even more. No major differences were observed between day 24 and day 30 concerning vanillin. The highest amounts of cinnamic acid were detected in the roots harvested on the 12th, 15th, 18th, and 21st days (26.98 - 28.87 µg g^{-1}), while the lowest values were found in the roots harvested on the 1st, 3rd, 27th and 30th days (4.02 - 5.97 µg g^{-1}). The highest levels of rosmarinic acid were detected between days 15 and 24, with an approximately 5-fold increase compared to day 1. It decreased dramatically from 56.79 µg g^{-1} on day 24 to 17.37 µg g^{-1} on day 27 and to 7.89 µg g^{-1} on day 30. Chlorogenic acid reached its highest levels on day 12 (2771.31 µg g^{-1}) and day 15 (2739.13 µg g^{-1}). The decrease in the amount of chlorogenic acid from day 18 continued gradually until day 30 and reached its lowest value on day 30 (1619.15 µg g^{-1}).

As a result of the study, it was concluded that the 15-day culture period after the application of 1 mM MeJA was the optimal culture period for phenol production in the adventitious roots of *H. niger*. Root growth parameters studied were root fresh weights, root growth indexes, and root dry weights. There were statistically significant differences in all growth parameters depending on the culture times ($p \leq 0.05$). The lowest fresh weights were found in roots harvested on the 1st and 3rd days after the application of 1 mM MeJA (4.17-4.24 g 100 ml^{-1}). Fresh weights increased significantly on days 6, 9, and 12 of culture with values of 5.04, 5.79, and 8.53 g 100 ml^{-1}, respectively (Figure 2A). However, no significant differences were observed between harvests grown at 3-day intervals from day 12 to day 30. The root growth index increased slowly from day 3 and peaked (1.05) on day 12 (Figure 2B). Thereafter, the growth index remained constant until day 30, with no significant differences. Root dry weights were lowest on days 1 and 3 and increased significantly on days 6, 9, and 12, similar to the trends in fresh weight and growth index. While root dry weights varied between 0.80 and 0.82 at all culture times, there were no significant differences between days 12 and 30 (Figure 2C). The lowest root dry weights ranged from 0.39 to 0.42 g 100 ml^{-1} and the highest root dry weights ranged from 0.80 to 0.83 g 100 ml^{-1}. Based on these results, it was found that the optimal values in terms of root growth parameters were obtained for roots cultured during 12, 15, 18, 21, 24, 27, and 30 days.

Table 1. Effects of culture times on the phenolic compounds in *H. niger* adventitious roots stimulated with 1 mM MeJA.

Culture times (day)	TPC (mg g^{-1})	Gallic acid (µg g^{-1})	Catechin (µg g^{-1})	Epicatechin (µg g^{-1})	Vanillin (µg g^{-1})	Cinnamic acid (µg g^{-1})	Rosmarinic acid (µg g^{-1})	Chlorogenic acid (µg g^{-1})
1	5.59 g*	24.75 ef	97.43 d	6.49 cd	15.48 c	4.02 d	10.08 ef	1690.54 ef
3	6.37 g	39.53 cd	126.90 d	6.54 cd	31.84 b	5.26 d	12.97 e	1889.99 de
6	10.97 de	54.05 b	133.12 d	9.67 c	37.84 a	12.97 c	23.63 c	2135.17 cd
9	11.42 d	62.21 a	307.81 c	9.38 c	40.01 a	20.98 b	26.08 c	2240.32 bc
12	15.68 c	62.58 a	485.62 b	15.08 b	39.12 a	26.98 a	35.02 b	2771.31 a
15	19.33 a	61.92 a	598.77 a	28.02 a	38.49 a	27.63 a	56.98 a	2739.13 a
18	17.16 bc	52.91 a	596.10 a	4.86 de	14.15 c	28.49 a	54.58 a	2494.44 b
21	17.45 b	49.90 bc	598.83 a	2.88 ef	4.13 d	28.87 a	55.78 a	2269.01 bc
24	11.71 d	34.34 de	500.66 b	0.00 f	4.24 d	12.60 c	56.79 a	2116.99 cd
27	9.68 ef	21.16 f	474.39 b	0.00 f	4.25 d	5.97 d	17.37 d	1919.85 de
30	8.66 f	18.05 f	459.43 b	0.00 f	4.26 d	5.56 d	7.89 f	1619.15 f

* Differences between means indicated by the same letters are not statistically significant (Duncan's multiple range test, $p \leq 0.05$)

4. DISCUSSION and CONCLUSION

In this study, the effects of different culture times on *H. niger* adventitious root cultures treated with 1 mM MeJA were evaluated in terms of root growth parameters and secondary metabolite accumulation. Root growth parameters, including root fresh weight, root growth index, and root dry weight, showed a similar pattern. Application of 1 mM MeJA resulted in no change in root growth on days 1, 3, and 6. However, on the 9th day, roots started to grow rapidly and reached their maximum growth on the 12th day. On the 12th day, root growth stopped and there was no statistically significant difference on the 12th, 15th, 18th, 21st, 24th, 27th, and 30th days. Similar to our study, it was reported that when 0.1 mM MeJA was applied to *Salvia miltiorrhiza* root cultures, root growth increased steadily until the 12th day and reached its maximum on the 12th day (Xiao *et al.,* 2009). It is known that there are differences in root growth as a function of MeJA concentration and culture time, as indicated by previous studies on different plants. Treatment of root cultures of *Glycyrrhiza glabra* (Shabani *et al.,* 2009) and *Setaria parviflora* (Kang *et al.,* 2004) with high concentrations of MeJA negatively affected root growth, and root growth decreased to the lowest level 24 and 72 h after application of 2 mM MeJA. In root cultures of *Datura metel*, root growth after application of various elicitors remained in the lag phase for the first 6 days, increased linearly for the next 21 days, and stopped after day 27 (Ajungla *et al.,* 2009). Application of chitosan in *Hypericum perforatum* root cultures (Brasili *et al.,* 2016) and application of pectinase in *Catharanthus roseus* root cultures (Rijhwani and Shanks 1998) suppressed root growth compared to the control group. Considering the results of the previous studies and this study, it is concluded that the effects of harvest periods on *in vitro* production of secondary metabolites vary considerably depending on the plant species, application of the elicitor, type of elicitor, and concentration of the elicitor. In particular, the application of elicitors that act as signaling molecules in the plant defense system, such as MeJA, inhibits the mitotic cycle in plant cells and reduces cell division and biomass increase by stopping cell division in G1 phase before entering S phase. For this reason, biomass production decreases or even stops after high concentrations of MeJA (Ho *et al.,* 2018; Kang *et al.,* 2004; Mendoza *et al.,* 2018).

This study also examined the effects of culture duration on the accumulation of hyoscyamine and scopolamine in adventitious roots of *H. niger*. Genotypes, metabolites, and elicitor applications have a great impact on determining the most appropriate culture time to achieve high efficiency *in vitro* production of secondary metabolites. MeJA is one of the most important elicitors used to enhance secondary metabolite production, and MeJA is known to significantly affect alkaloid production, especially as a function of culture time (Kang *et al.,* 2004; Zayed & Wink 2005). The 3-day application of MeJA to the roots of *Scopolia parviflora* significantly affected the amount of hyoscyamine and scopolamine as a function of MeJA concentration and culture time (Kang *et al.,* 2004). In the same study, MeJA was reported to regulate the synthesis of the enzymes putrescine N-methyl transferase (PMT) and hyoscyamine-6β-hydroxylase (H6H), thereby increasing alkaloid production. In *Peganum harmala*, the highest accumulation of alkaloids occurred in roots harvested on day 5 after MeJA application (Zayed & Wink 2005). Lee *et al.,* (2001) found that the alkaloid content in *Atropa belladonna* hair roots treated with 2 mM salicylic acid decreased by 35% on day 1, 80% on day 3, and 90% on day 7. The researchers explained the reason for this decrease as an increase in the amount of alkaloids entering the culture medium, explaining that salicylic acid accelerates the transfer of alkaloids from the roots to the culture medium (Lee *et al.,* 2001). In transformed A. belladonna hairy roots after phytosulfokine-α (PSK-α) application, the highest alkaloid production occurred in 4 weeks (Sasaki *et al.,* 2002). The optimal culture time for alkaloid production in root cultures varies considerably depending on elicitor application and genotypes, as previously reported (Harfi *et*

al., 2016; Moharrami *et al.,* 2017). The changes in phenolics in randomly selected roots after application of 1 mM MeJA were also investigated.

After the application of 1 mM MeJA, the changes in phenolics in adventitious roots grown at intervals of 3 days to 30 days were also studied. It was found that TPC varied significantly depending on the culture period. The highest TPC value was obtained in the roots harvested on day 15. The extensive literature search revealed that studies on alkaloid-rich plant species such as *H. niger* focused on alkaloid production under *in vitro* conditions, while phenolic compounds were not the main focus. However, *H. niger* is also rich in very valuable phenolics for the pharmaceutical and cosmetic industries. In studies investigating phenolic content as a function of culture time after biotic or abiotic elicitor applications, the highest total phenolic accumulation in hairy roots of *Harpagophytum procumbens* stimulated with *Agrobacterium rhizogenes* was found in roots collected on day 21 (Georgiev *et al.,* 2006). In cell cultures of *Thevetia peruviana* treated with MeJA, the highest total phenolic content was reached at the 96th hour (Mendoza *et al.,* 2018). The application of 100 µM cadmium to *Vaccinium corymbosum* caused the highest accumulation of total phenolic content on day 21 (Manquian-Cerda *et al.,* 2016). Culture time was shown to significantly alter phenolic content after elicitor applications on various plants such as *Pelargonium sidoides* (Yousefian *et al.,* 2020), *Polygonum hydropiper* (Ono *et al.,* 1998), *Capsicum frutescens* (Suresh & Ravishankar 2005), *Salvia miltiorrhiza* (Xiao *et al.,* 2009), *Agastache rugose* (Lee *et al.,* 2008), *Echinacea purpurea* (Demirci *et al.,* 2020). Ono *et al.,* (1998) reported that the changes in phenolic content during the culture period were due to the conversion of some phenols to other compounds. The researchers found that the amount of catechin, which increased until day 6, began to decrease later, which was due to the catechin being converted to procyanidin by bioconversion.

In the *in vitro* production of secondary metabolites, one of the most important reasons for the decrease in secondary metabolites after reaching the highest level in plant cells and tissues due to the increase in culture time is the increased release of metabolites from the explants to the culture medium (Lee *et al.,* 2001). Therefore, the accumulation of metabolites in plant materials decreases while it increases in culture media. In addition, conversion of metabolites to another compound by biodegradation or degradation of elicitor substances added to culture media to increase metabolite yield are other reasons for the decrease in secondary metabolite accumulation during culture (Ono *et al.,* 1998; Suresh and Ravishankar 2005). From the results, harvesting periods for *in vitro* production of secondary metabolites are the most important criterion for the production of large amounts of metabolites. However, many factors such as genotype, metabolite, application of elicitors, etc., play a role in determining the most appropriate culture time.

In this study, the effects of culture time on root growth, tropane alkaloids, and phenolics production in *H. niger* adventitious root cultures treated with 1 mM MeJA were investigated, and it was found that culture time significantly affected root growth and secondary metabolites production. Roots collected on day 15 after MeJA application was found to be the optimal culture time in terms of root growth and secondary metabolite content. However, further research is needed due to the lack of appropriate work to determine the optimal time of culture to increase yield in secondary metabolite production in plants rich in tropane alkaloids and *H. niger*. This work was the first to investigate the effects of culture time on highly efficient secondary metabolite production and optimal root growth in *H. niger* adventitious root cultures. Future experiments will use bioreactor systems in which pH, soluble oxygen, and culture media can be controlled to recover high levels of secondary metabolites from *H. niger* adventitious root cultures. The results of this study provide important guidance for future scale-up production using bioreactors to recover large amounts of alkaloids and phenols.

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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

Hikmet Deveci: Formal analysis, Investigation. **Tunhan Demirci:** Conceptualization, Methodology, Formal analysis, Investigation, Writing- original draft, Writing-review & editing, Supervision. **Nilgun Gokturk Baydar:** Writing- review & editing, Supervision.

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Research Article

Antioxidant properties of *Lycianthes rantonnetii* and contents of vitamin and element

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Abstract: The aim of this study was to investigate the antioxidant, antiradical activity of the *Lycianthes rantonnetii* (Solanacaeae) plant, to determine levels of element (Fe, Zn, Mn, Cu, Cr, Mo, Cd, As, Pb, Co, V, Tl, Li, Ti, Sr, Be), mineral (Mg, P) and vitamin (A, E, C). In the study, metals and mineral analyses were carried out using dry ashing method with ICP-OES, vitamin A and vitamin E analyses were carried out using the HPLC method and vitamin C, total phenol, flavonoids, antioxidant capacity, DPPH, ABTS, hydrogen peroxide, hydroxyl radicals scavenging activity and anti-hemolytic activity of the methanol extract *Lycianthes rantonnetii* (*L. rantonnetii*) plant was determined spectrophotometrically. According to the results, Cu, Zn, Co, Mg, retinol, α-tocopherol, ascorbic acid, total antioxidant activity, phenolic and flavonoid contents of *L. rantonnetii* were 57.60 ± 4.83 µmol/kg, 0.14 ± 0.01 mmol/kg, 2.23 ± 0.10 µmol/kg, 39.13 ± 1.76 mmol/kg, 12.22 ± 3.37 µmol/kg, 104.55 ± 7.44 µmol/kg, 80.61 ± 9.31 mg/100 g, 11.45 ± 0.60 mM ascorbic acid/g, 5.33 ± 0.41 mg GA/g and 3.76 ± 0.29 mg QE/g, respectively. The results of this study showed that the content of antioxidant vitamins, minerals of the *L. rantonnetii* plant was at high levels and it contains a reasonable amount of total phenol, and flavonoids also, it was determined that the plant had a high scavenging activity for free radicals. It is believed that *L. rantonnetii* can be used as an additive for food products in the various food sectors with appropriate antioxidant activities.

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1. INTRODUCTION

Nature is permanently a vital and abundant origin of various health-promoting ingredients. Some of these natural products contain fruit, vegetables, herbs, and spices that are widely used. Therefore, there are many medicinal plants that have shown important potential for health enhancement. The potential antioxidant properties of natural sources are one of the most useful impacts (Slavin & Lloyd, 2012).

Solanaceae family also called the nightshade or potato family, is one of the biggest and economically most significant families of angiosperms, including significant food, spice, and

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medicine plants, comprises of around 90 genera and 3000 species. Nearly 50% of the species in the family are included in the widespread, morphologically diverse, and economically significant genus Solanum. The family is almost cosmopolitan in distribution, discovered in entire tropical and temperate districts, but with a concentration of variety in Latin America and Australia. (D'Arcy, 1979; Jacobs & Eshbaugh, 1983).

Trace elements play an important role in the metabolic regulation of the human body and the excess of elements in food in the etiology of some diseases (Eken *et al*., 2017). Medicinal plants containing toxic elements such as Pb, Cd and As can have a harmful effect (Güvenç *et al*., 2007).

Reactive oxygen species (ROS) is produced as a natural direct consequence of regular oxygen metabolism. In common, harmful effects such as DNA damage, polyunsaturated fatty acid oxidation in lipids, and amino acid oxidation in proteins oxidatively prevent the growth of particular enzymes by co-factor oxidation (Khan *et al*., 2018). Free radicals affect the formation of atherosclerosis, ischemic heart diseases, old age, inflammation, diabetes, neurodegenerative diseases, and many other diseases (Jain *et al*., 2008).

The oxidant-antioxidant balance is related to maintaining a balance between the useful and damaging results of ROS (Gutteridge, 1995). Both endogenous and exogenous antioxidants are active against oxidative stress, and endogenous antioxidants cannot fully protect cells from ROS (Tanaka *et al*., 2011). A significant source of natural antioxidants could be the use of medicinal plants (Chandra *et al*., 2013), including anthocyanins, flavonoids, flavones, isoflavones, coumarins, catechins, and vitamins C and E, β-carotene and α-tocopherol, which are considered to have possible antioxidants (Jain *et al*., 2008).

The aim of this study was to analysis the antioxidant and antiradical activities in methanol extracts of *L. rantonnetii* by different analytical methods; total antioxidant activity**,** DPPH, ABTS, hydrogen peroxide, and hydroxyl radicals scavenging activity and anti-hemolytic activity, total phenolic and flavonoid contents, and, in addition, to investigate elements (Fe, Zn, Mn, Cu, Cr, Mo, Cd, As, Pb, Co, V, Tl, Li, Ti, Sr, Be), mineral (Mg, P) and vitamin (A, E, C).

2. MATERIAL and METHODS

2.1. Plant Material and Extraction

Lycianthes rantonnetii (Carrière ex Lesc.) Bitter (Solanacaeae) plant used in the study was collected from Ranya city, Sulaymaniyah, Iraq, on 10 July 2019. Plant species identification was made by Prof. Dr. Fevzi ÖZGÖKÇE, University of Van Yüzüncü Yil, Science Faculty, Biology Department. Witness plant specimen, stored with the code VANF-164103 in Van Yüzüncü Yil University Herbarium. The plant samples were dried in the shade without exposure to sunlight and stored for analysis. After drying the plant in the shade, crushed it to powder, 20 g of the powdered plant was weighed and transferred to a colored bottle, and 400 ml of 75% methanol was added. The sample stored in the colored bottle was extracted for 48 hours at room temperature with stirring. The solution is then separated from the precipitate by filtration. The methanol in the filtrate was removed using a rotary evaporator and aqueous extracts were obtained by lyophilization with a freeze-drier (-86 °C).

2.2. Determination of Vitamin (A, E)

2.2.1. Standard solutions and calibration

α-tocopherol and all-trans-retinol stock solutions were prepared at 500 µg/mL in methanol. Stock solutions were appropriately diluted with the mobile phase for the preparation of the standard solutions. Calibration was calculated by linear regression analysis of the peak area versus the concentrations of the standard solutions.

2.2.2. Extraction procedure

In our study, vitamin A and vitamin E amounts of *L. rantonnetii* plant were determined by modifying the method used by (Sahin *et al*., 2005; Al-Saleh *et al*., 2006). From the plant samples which were dried and ground in the shade, 5 g was weighed and extracted with n-hexane and ethanol. 0.01 % BHT was added to them, vortexed, and kept in the dark for 24 hours. Then centrifuged at +4 °C and 4000 rpm for 10 minutes. The supernatant was filtered using Whatman filter paper and 0.5 mL of n-hexane was added and dried at 37 °C under nitrogen gas. After drying, the residue was dissolved in a mixture of 200 µL methanol+tetrahydrofuran and made ready for analysis.

2.2.3. Chromatographic conditions

Analyzes, Gl Science C_{18} reverse-phase HPLC column (250 x 4.6 mm ID), methanol+tetrahydrofuran (80:20) mobile phase, at a flow rate of 1.5 mL/min, at a temperature of 25 °C. Thermo Scientific Finnigan Surveyor model in high-performance liquid chromatography, using a PDA array detector, applications in a volume of 100 µL in dark-colored vials in tray autosampler (-8 °C) were performed at 325 and 290 nm (α-tocopherol and all-trans-retinol). The chromatographic analysis was performed at 40 ºC with isocratic elution.

2.3. Vitamin C Analysis

Vitamin C stock solution was prepared at 4000 mg/mL in metaphosphoric acid. The stock solutions were appropriately diluted correctly with double distilled water to prepare standard solution preparation. Calibration was calculated by linear regression analysis of the absorbance versus the concentration of the standard solution. Vitamin C content of *L. rantonnetii* was measured spectrophotometrically (Shimadzu UV 1800, Japan) at 521 nm using 2,4-dinitrophenyl hydrazine method.

2.4. Element Determination

Detection of mineral content of *L. rantonnetii* plant was performed by using dry ashing method. 1 g of sample was weighed and placed in the crucible, put in an oven for 4-5 hours at 105 °C, the dried samples were crushed in thoroughly cleaned porcelain mortars. 2 mL of ethyl alcohol-sulfuric acid mixture (95:5) was added to each sample. Then it was left in the ash furnace set at 250 °C. The temperature was increased by 100 °C per hour until the temperature of the oven reached 550 °C. 5 mL of prepared 3N hydrochloric acid solution was added on the samples taken out from the ash furnace and it was completed to 50 mL with distilled water. The metal analysis was conducted using multi-element (NIST SRM) reference materials (inorganic ventures IV-Stock-1643). The concentration ranges of the standards for the elements (0.1-7 mg/L). Element analysis of P, Mg, Fe, Zn, Cu, Cr, Mn, Mo, Cd, As, Pb, Co, V, Tl, Li, Ti, Sr and Be were used inductively coupled plasma-optical emission spectrometry. ICP-OES (Thermo ICP-OES iCAP 6300 Duo, England) with ASX-520 autosampler was used for the analyses. Its operating conditions are reported as; spectral range (166-847 nm), plasma viewing (Dual), Plasma and shear gas (Argon), Nebulizer (Concentric glass), sample flush time (40 seconds), analysis pump rate (50 rpm), auxiliary gas flow (0.5 L/min), nebulizer gas flow (0.65 L/min), RF power (1150 W).

2.5. Determination of Total Phenol Content

The total phenol content of *L. rantonnetii* was carried out by utilizing reagent (Folin-Ciocalteu) (Yi *et al*., 1997; Gamez-Meza *et al*., 1999). 0.3 mL of 20 % Na_2CO_3 was added to the plant samples prepared by diluting with methanol, then combined with 100 µL of water diluted Folin reagent (1:1) and 2 hours incubated at room temperature. At 765 nm wavelength, samples absorbance read against a control sample. The phenol contents are expressed as gallic acid equivalents per gram (mg GAE/g) of extract.

2.6. Determination of Total Flavonoid Content

In this study, in order to determine the flavonoid content of the extract of *L. rantonnetii*, 0.1 mL of $Al(NO_3)_3$ solution mixed with 0.2 mL of the samples diluted with methanol and incubated at room temperature for 40 min. At 415 nm wavelength samples absorbance was recorded versus the control sample (Urgeova & Polivkau, 2009). Flavonoid concentration was given as mg/g equivalent of quercetin.

2.7. Determination of Total Antioxidant Capacity

In this study, the method progressed by (Prieto *et al*., 1999) was used to detect the total antioxidant capacity of *L. rantonnetii* plant. The assay depends on the reduction of acidic Mo (VI) to (V) at acidic pH which leads to the form green phosphate/Mo (V) complex. Added 0.2 mL of reagent (0.6 M, 28 mM, and 4 mM of sulfuric acid, sodium phosphate, and ammonium molybdate, respectively) to 0.2 mL samples of different concentrations of plant extract diluted with methanol, and kept for 90 minutes at 95 °C. Before reading the absorbance of samples versus the control sample at 695 nm wavelength, the samples were cooled to room temperature. Antioxidant capacity was given as mM ascorbic acid/g.

2.8. DPPH Radical Scavenging Capacity

DPPH radical scavenging capacity determination assay depends on the principle of spectrophotometric determination of the characteristic purple lightening by scavenging the stable DPPH free radical, by these chemicals in the presence of antioxidant chemicals that donate electrons or hydrogen atoms (Cuendet *et al*., 1997; Chen *et al*., 2009). In this study, in order to determine *L. rantonnetii* ability to scavenge DPPH radical, 5 mL of 0.004% DPPH solution was added to the methanol diluted different concentrations of plant extract and incubated for 30 min. the absorbance was measured against a blank at 517 nm. Antioxidant activity of the samples was expressed in term of IC_{50} values (µg/mL required to inhibit the formation of DPPH radicals by 50%) calculated from the graph in which the percentage inhibition was plotted against extract concentration. The synthetic antioxidant BHT was included in experiments as a positive control. The percentage inhibition of the free radical DPPH was calculated according to the following formula.

$$\text{Inhibition (\%)} = \left\{ \frac{A_{Blank} - A_{Sample}}{A_{Blank}} \right\} X\ 100$$

2.9. ABTS Radical Scavenging Capacity

In this study, The ABTS radical scavenging effect of *L. rantonnetii* plant was done with 0.1 M phosphate buffer with pH 7.4. Prepared 2 mM ABTS solution with 2.45 mM solution of potassium persulfate. Then these two solutions were prepared mixed up by magnetic stirrer at room temperature/dark for 12-16 hours. The absorbance of the prepared solution was recorded at 734 nm. The strength of molecules to scavenge stable free radicals was done with a synthetic antioxidant (Trolox) (Arnao *et al*., 2001).

$$\text{Inhibition (\%)} = \left\{ \frac{A_{Blank} - A_{Sample}}{A_{Blank}} \right\} X\ 100$$

2.10. Hydroxyl Radical Scavenging Capacity

In this study, in determining the hydroxyl radical removal capacity of *L. rantonnetii* plant extract, the hydroxyl radicals formed by the (Fe^{+3}/ascorbate/EDTA/H_2O_2) system. The method

based on the spectrophotometric measurement of thiobarbituric acid, reactive products released by the degradation of deoxyribose (Kunchandy & Rao, 1990). *L. rantonnetii* plant extract was added to the samples prepared in different concentrations from 3.0 mM deoxyribose, and 1 mM for each of the $FeCl_3$, EDTA, ascorbic acid, and H_2O_2 solutions, after that added 20 mM phosphate buffer (pH 7.4) until the volume reached 1 mL, kept the reaction for 1 hour at 37 °C. Before recording the absorbance added 1 mL for both the TBA, and TCA, and boiled for 30 minutes. The absorbance at 532 nm wavelength of the colored complex formed by TBA of the released malondialdehyde was read against the control sample. The IC_{50} values were determined by plotting the % inhibition values obtained against different concentrations of the plant extract.

$$\text{Inhibition (\%)} = \left\{ \frac{A_{Blank} - A_{Sample}}{A_{Blank}} \right\} X\ 100$$

2.11. Determination of Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide (H_2O_2) scavenging activity of *L. rantonnetii* plant methanol extract was measured spectrophotometrically at 230 nm. For this, phosphate buffer (50 mM, pH 7.4) utilized to preparative 43 mM of H_2O_2 solution. *L. rantonnetii* plant extracts obtained from different concentrations were used. The volume of plant extract and standard antioxidant substances used in the study BHT solutions completed with buffer solution up to 0.4 ml. Before incubation for 10 minutes at the normal temperature inside the room added 0.6 ml of H_2O_2 solution. The decreasing amount of H_2O_2 at 230 nm was read as the reduced absorbance (Ruch *et al*., 1989).

2.12. Phenylhydrazine method

In this method. 1 mL phenylhydrazine, 0.1 mL 20% PCV, 1.85 mL buffer were added to the samples prepared with different concentrations of the plant methanol extract. Then, before centrifuging them for 10 min at 4000 rpm incubated at 37 °C for 1 hr. Finally, the supernatant portion was transferred to other tubes; the absorbance at 540 nm was read against the control sample (Valenzuela *et al*., 1985).

3. RESULTS

Total antioxidant capacity, total phenolic and total flavonoid contents, the radical scavenging activities of DPPH, ABTS, hydroxyl radical, and hydrogen peroxide as well as its anti-hemolytic activity were measured to determine the antioxidant properties of *L. rantonnetii*. In addition, the levels of vitamin, element, and minerals in *L. rantonnetii* were determined and the results were shown in Table 1 and 2.

Table 1. Vitamin A, E, C, total antioxidant capacity, total phenolic and flavonoid, element (P, Mg, Fe, Zn, Cu, Cr, Mn, Mo, Cd, As, Pb, Co, V, Tl, Li, Ti, Sr and Be) content in L. rantonnetii.

Parameters	*Lycianthes rantonnetii* $\bar{X}$ ± SEM
α-tocopherol (µmol/kg)	104.55 ± 7.44
Retinol (µmol/kg)	12.22 ± 3.37
Vitamin C (mg/100g)	80.61 ± 9.31
Total phenolic content (mg GA/ g)	5.33 ± 0.41
Total flavonoid content (mg QE/g)	3.76 ± 0.29
Total antioxidant capacity (mM A.A/ g)	11.45 ± 0.60
Be (µmol/kg)	0.09 ± 0.00
Li (µmol/kg)	0.66 ± 0.29
V (µmol/kg)	1.57 ± 0.13
Ti (µmol/kg)	3.07 ± 0.26
Cr (µmol/kg)	4.61 ± 0.33
Cu (µmol/kg)	57.60 ± 4.83
Sr (µmol/kg)	14.41 ± 3.24
As (µmol/kg)	0.67 ± 0.01
Tl (µmol/kg)	0.16 ± 0.01
Cd (µmol/kg)	0.75 ± 0.02
Co (µmol/kg)	2.23 ± 0.10
Pb (µmol/kg)	0.83 ± 0.11
Mo (µmol/kg)	1.14 ± 0.01
Mg (mmol/kg)	39.13 ± 1.76
Fe (mmol/kg)	0.63 ± 0.01
Mn (mmol/kg)	0.42 ± 0.02
P (mmol/kg)	34.60 ± 0.42
Zn (mmol/kg)	0.14 ± 0.01

Values are expressed as mean ± standard error of the mean ($\bar{X}$ ± SEM). Samples were carried out in triplicate.

Table 2. Values of % Inhibition and IC_{50} (µg/mL) in methanol extracts of *L. rantonnetii* compared with a positive controls.

	Control	% Inhibition $\bar{X}$ ± SEM	IC_{50} (µg/mL) $\bar{X}$ ± SEM
DPPH$^{\bullet}$		67.25 ± 0.08	96.52 ± 0.13
	BHT	74.78 ± 3.04	66.04 ± 13.55
$OH^{\bullet}$		61.18 ± 1.39	32.46 ± 0.81
	BHT	81.32 ± 0.13	84.53 ± 6.03
H_2O_2		54.72 ± 0.13	34.32 ± 0.05
	BHT	54.17 ± 0.03	23.57 ± 0.29
ABTS		57.75 ± 1.11	112.81 ± 0.41
	Trolox	96.56 ± 0.01	53.11 ± 0.70
$PhNHNH_2$		67.18 ± 7.33	191.13 ± 52.51
	Trolox	50.77 ± 0.58	16.17± 6.22

Values are expressed as mean ± standard error of the mean ($\bar{X}$ ± SEM). Samples were carried out in triplicate. DPPH: 2,2-diphenyl-1-picrylhydrazyl, OH**:** Hydroxyl, H_2O_2**:** hydrogen peroxide, ABTS: 2.2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), $PhNHNH_2$: Phenylhydrazine.

Figure 1 and 2 shows at 325 and 290 nm α-tocopherol and all-trans-retinol chromatograms**.** While evaluating the results, it was observed that *L. rantonnetii* contained vitamins A, and E in significant levels.

Figure 1. Chromatogram of the vitamin A (mobile phase: methanol/tetrahydrofuran (20/80 v/v), flow rate: 1.5 mL min^{-1}. Column: GI Science C18 5µL (250/4.6 mm), wavelength: 325 nm).

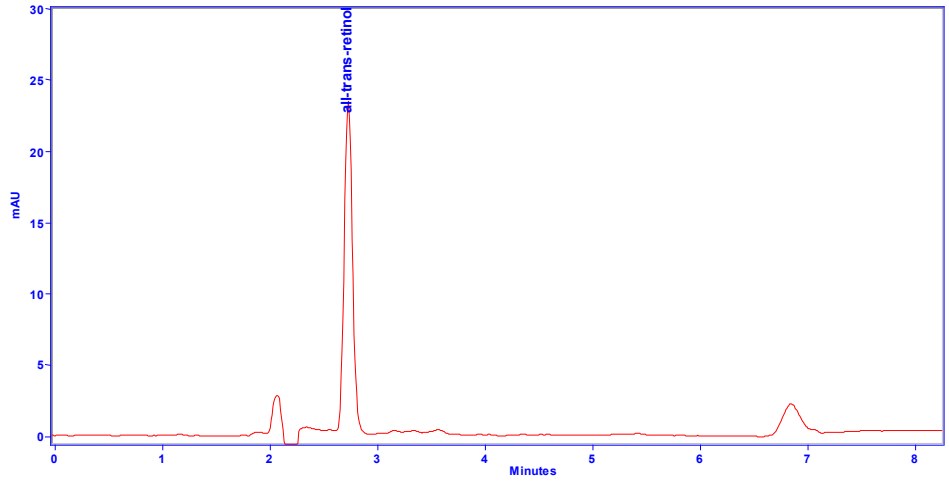


Figure 2. Chromatogram of vitamin E (mobile phase: methanol/tetrahydrofuran (20/80 v/v), flow rate: 1.5 mL min^{-1}. Column: GI C18 5µL (250/4.6 mm), wavelength: 290 nm).

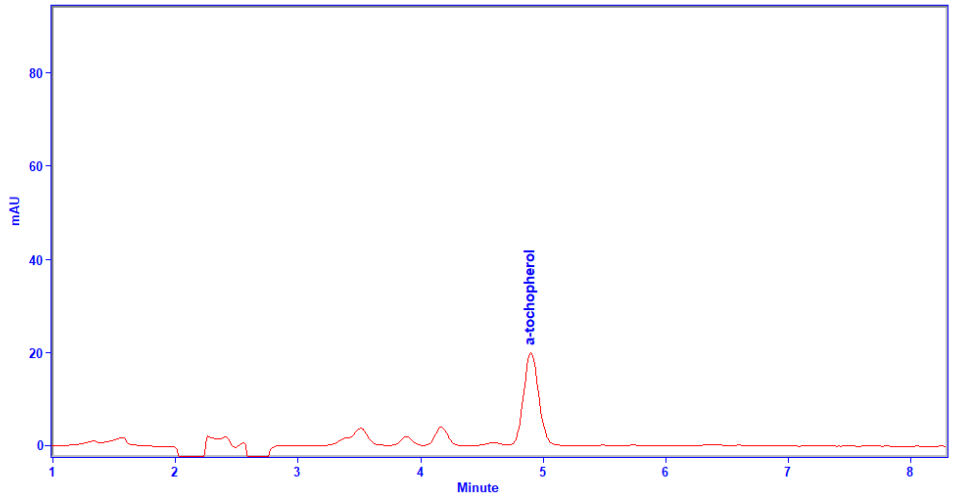


Figure 3 shows % inhibition and IC_{50} values of DPPH radical and BHT and ABTS radical for *L. rantonnetii* and trolox.

Figure 3. % inhibition and IC_{50} values of DPPH radical and BHT and ABTS radical for *L. rantonnetii* and trolox.

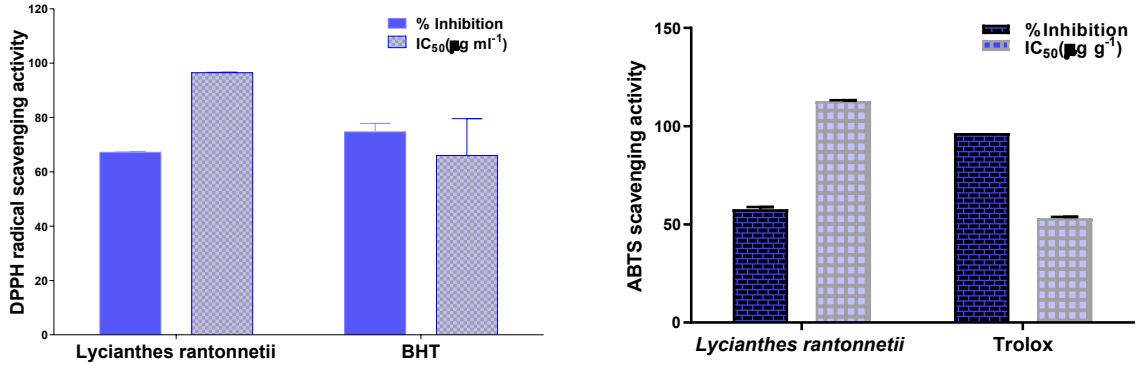


Figure 4 demonstrates % inhibition and IC_{50} values of the hydroxyl radical scavenging activity and BHT and hydrogen peroxide radical scavenging activity and BHT of *L. rantonnetii*

Figure 4. Graph showing the change in % inhibition and IC_{50} values of the hydroxyl radical scavenging activity and BHT and hydrogen peroxide radical scavenging activity of *L. rantonnetii* and BHT.

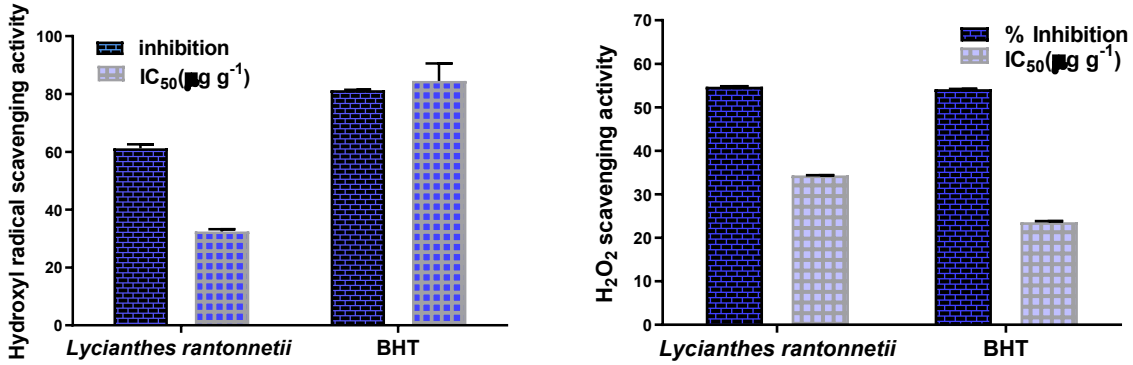
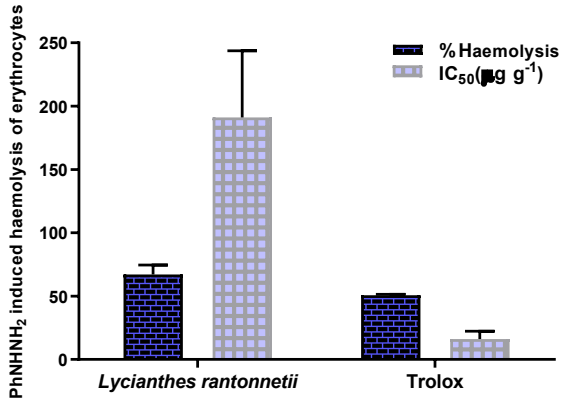


Figure 5 illustrated % haemolysis and IC_{50} values of the phenylhydrazine for *L. rantonnetii* plant and trolox.

Figure 5. Graph showing the change in % haemolysis and IC_{50} values of the phenylhydrazine for *L. rantonnetii* plant and trolox.



4. DISCUSSION and CONCLUSION

Oxidative stress occurs when an increase in ROS levels affects the balance between ROS development and detoxification and contributes to impaired cellular activity (Imlay, 2003). Therefore, many defense mechanisms, including various antioxidants and detoxifying enzymes, must regulate ROS compounds (Duarte & Lunec, 2005). Deficiencies or degradation in these antioxidants could contribute to oxidative stress, which can trigger cell harm and lysis (Valko *et al*., 2007). The pathways observed by antioxidant protection are; preventing the formation of free radicals, scavenging of oxidants, turning toxic free radicals into less toxic compounds, disrupting the production of secondary toxic metabolites and inflammatory mediators, restricting the secondary oxidants chain propagation, restoring and initiating harmed molecules, and improving the endogenous antioxidant synthetic (Halliwell, 2007).

In this work, first of all, the amount of vitamins A, E, and C in *L. rantonnetii* plant was determined. In terms of minimizing the risk of illness and develop positive health, vitamins play a significant part. Vitamins are necessary for all splitting body cells to operate normally and proliferate. (Piccardi & Manissier, 2009).

The vitamin C level of *L. rantonnetii* plant extract included in the study was determined as 80.61 ± 9.31 mg/100g. The vitamin A level was determined as 12.22 ± 3.37 μmol/kg and the vitamin E level as 104.55 ± 7.44 μmol/kg. In the literature review, no study was found on the levels of vitamins A, C, and E of *L. rantonnetii* plant. At the same time, there is no study on these vitamin levels in *Lycianthes* species. However, the vitamin C level of *Capsicum* genus (chili pepper) plant, are important species belonging to the Solanaceae family, also has the same tribe with *L. rantonnetii* plant, was determined to be 27.13 ± 0.05 mg/100g. In a study conducted by Emmanuel-Ikpeme *et al*., the vitamin E level of the *Capsicum* genus (chili pepper) plant is 0.41 ± 0.02 mg/100g, and the vitamin A level is 1.26 ± 0.03 μg/100g (Emmanuel-Ikpeme *et al*., 2014). When the *L. rantonnetii* plant was compared with the chili pepper plant, it was determined that the vitamin C, A, and E levels were higher than the chili pepper plant. This shows that the plant is a better source of vitamins than the chili pepper plant. Considering the results, it was determined that the *L. rantonnetii*. Plant had a significant vitamin A, C, and E content, and especially vitamin C and E were at a high level.

Polyphenols are organic compounds found abundantly in the plant (Lecour & Lamont, 2011). They are necessary for plant growth, reproduction, color, and aroma formation (Guiné *et al*., 2015). Polyphenols consists of phenolic compounds, phenolic acids, and flavonoids (Ratnam *et al*., 2006). These compositions have various pathways for practicing their antioxidant powers, including free radical scavenging, strengthening endogenous enzymatic protections, reducing lipoperoxidation (Watson *et al*., 2014). The antioxidant function of some phenolic substances in the work by (Servili *et al*., 2014), depends largely on their degree of hydroxylation. They also act as H atom donors, singlet oxygen, and superoxide radical scavenger, and metal chelator (Heleno *et al*., 2015). It is well-established that oxidative processes are related to diverse pathologies (Watson *et al*., 2014), polyphenols contribute to the prevention of cardiovascular disease, cancer and osteoporosis reduction and play a role in combating neurodegenerative diseases and diabetes mellitus (Scalbert *et al*., 2005).

In this study, total phenol, total flavonoid, and total antioxidant capacities of *L. rantonnetii* plant were determined. The total phenol amount was determined using the gallic acid standard curve graph, and the total phenol amount of the plant was calculated as 5.33 ± 0.41 mg gallic acid/g. Total flavonoid contents were considered using the typical quercetin curve, and the total flavonoid content of the plant was determined as 3.76 ± 0.29 mg quercetin/g. Furthermore, the overall antioxidant potential was calculated in line with the standard for ascorbic acid, and the total antioxidant value of the plant was 11.45 ± 0.60 mM/g ascorbic acid. These findings indicate that phenolic and flavonoid compounds are responsible for the higher rates of antioxidant activity.

In the literature review, no study was found on the total amounts of phenols and flavonoids of the *L. rantonnetii* plant. The overall amounts of phenols and flavonoids of methanol extract of *Capsicum* genus (chili pepper) was determined the phenol content; 480 mg gallic acid/g, flavonoid content; 240 mg quercetin/g (Helmja *et al*., 2007). The results indicate that the total phenol and flavonoid levels of *L. rantonnetii* plant were close to the chili pepper plant.

Elements are also essential to plants. If a plant cannot obtain an element from the soil, its life process is disrupted. Medicinal plants differ in trace element content as in all living organisms. All elements found in soil, air, and water enter the plant tissue, but in general, trace element intake varies according to the plant's need for an element, the amount of the element in the soil, and the soil type. The pharmacotherapy effects of pharmaceutical products obtained from plants are significantly affected by trace element factors in plant substances. Plants synthesize organic compounds in photosynthesis, including pharmacologically active, from various mineral constituents. More than 20 elements are involved in the synthesis of organic

compounds. Besides, all elements play important roles in the photosynthesis process (Suchacz & Wesołowski, 2006).

When the element and mineral levels of *L. rantonnetii* plant were examined in our study, it was found that it had content, 0.63 ± 0.01 mmol/kg Fe, 0.14 ± 0,01 mmol/kg Zn, 57.60 ± 4.83 µmol/kg Cu, 0.42 ± 0.02 mmol/kg Mn, 4.61 ± 0.33 µmol/kg Cr, 2.23 ± 0.10 µmol/kg Co, 39.13 ± 1.76 mmol/kg Mg, 34.60 ± 0.42 mmol/kg P, 14.41 ± 3.24 µmol/kg Sr, 3.07 ± 0.26 µmol/kg Ti, 1.57 ± 0.13 µmol/kg V, 1.14 ± 0.01 µmol/kg Mo, 0.83 ± 0.11 µmol/kg Pb, 0.75 ± 0.02 µmol/kg Cd, 0.67 ± 0.01 µmol/kg As, 0.66 ± 0.29 µmol/kg Li, 0.16 ± 0.01 µmol/kg Tl, 0.09 ± 0.00 µmol/kg Be.

Comparing the element and mineral levels of the *L. rantonnetii* plant, magnesium (Mg) > phosphorus (P) > iron (Fe) > manganese (Mn) > zinc (Zn) > copper(Cu) > strontium (Sr) > chromium (Cr) > titanium (Ti) > cobalt (Co) > vanadium (V) > molybdenum (Mo) > lead (Pb) > cadmium(Cd)> arsenic (As) > lithium (Li) > thallium (Tl) > beryllium (Be).

In the literature search, no study was found on the element and mineral levels of *L. rantonnetii* plant. However, in the studies conducted on the *Capsicum* genus (chili pepper), one of the important species belonging to the Solanaceae family, mineral levels were found to be 7l.4l ± 0.04 mg/100g Fe, 38.02 ± 0.05 ppm Zn, 18.22 ± 0.01 ppm Cu, 274.88 ± 0.02 mg/100g Mg, 13.27 ± 0.09 ppm Co, 28.75 ± 0.04 mg/100g P (Emmanuel-Ikpeme *et al*., 2014). Comparing *L. rantonnetii* plant to chili pepper plant, it was determined that Fe, Zn, Mg, and Co levels in chili pepper plant were higher, and Cu and P in *L. rantonnetii* plant were higher.

Spectrophotometric assays using DPPH and ABTS are the most popular methods for the *in vitro* evaluation of antioxidant ability in foods (Becker *et al*., 2019). The extract's free radical scavenging behavior was calculated using the constant free radical DPPH regarding hydrogen donation or radical scavenging capacity. Modifications that occur in the radical scavenging by hydrogen donations are attributable to the reaction between radicals and antioxidant molecules (Aadesariya *et al*., 2017). The sample concentration necessary to reduce the initial DPPH, ABTS concentration by 50% (IC_{50}) which calculated from inhibition plot under the experimental conditions are determined.

The scavenging capacity of the methanol extract of *L. rantonnetii* to scavenge DPPH, ABTS, and hydroxyl radicals, which are important free radicals, was determined, and the antiradical capacity of the plant was evaluated and compared with BHT, a synthetic antioxidant. The highest inhibition value of the DPPH radical of plant was determined as 67.25 ± 0.08% and 74.78 ± 3.04% for BHT. A smaller IC_{50} value means that antioxidant effect is greater. The specimen concentration in need to minimize the original concentration of DPPH, by 50 percent (IC_{50}) under the test conditions was calculated. For 96.52 ± 0.13 µg/mL and 66.04 ± 13.55 µg/mL for BHT, scavenging activity was recorded. It is safe to assume that *L. rantonnetii* plant's methanolic extract showed less potent antioxidant activity, with a lower inhibition percentage than BHT. In scavenging DPPH radical of plant was thus found to be less efficient than BHT.

In this study, the plant extract's scavenging capacity on ABTS radical was determined and compared with trolox, a synthetic antioxidant. The highest inhibition value of ABTS radical of plant was determined as 57.75 ± 1.11% and 96.56 ± 0.01% for trolox. A lower IC_{50} value means that antioxidant activity is larger. The specimen concentrations needed to decrease the initial ABTS intensity by 50 percent (IC_{50}) under the test conditions was calculated. Concentrations that inhibited the ABTS radical by 50 percent were determined as 112.81 ± 0.41 µg/mL in the plant and 53.11 ± 0.70 µg/mL for trolox. The inference can be drawn that the *L. rantonnetii* plant showed less potent antioxidant activity, with a lower inhibition ratio proportion than the BHT. Thus plant is less effective than trolox in scavenging ABTS radical.

In the study scavenging capacity of the hydroxyl radical of the *L. rantonnetii* plant was determined. The highest inhibition value of the plant extract's hydroxyl radical was determined as 61.18 ± 1.39% and 81.32 ± 0.13% for BHT. The concentrations that inhibit the hydroxyl radical by 50 percent were determined as 32.46 ± 0.81 µg/mL in plant and 84.53 ± 6.03 µg/mL for BHT. It has been determined that the plant is more effective than BHT in scavenging the hydroxyl radical. As a result of this study, it was found that *L. rantonnetii* plant effectively scavenges the hydroxyl radical.

Hydrogen peroxide is not a free radical but is called a ROS because it can be converted into other free radicals, such as hydroxyl radical, that facilitate much of the toxic results of H_2O_2 (Halliwell & Gutteridge, 1995). In this study, *L. rantonnetii* plant's hydrogen peroxide removal activity was also determined. Results indicated that the highest inhibition value of the hydrogen peroxide of both plant extract and BHT was determined, and the values close each other were 54.72 ± 0.13%, 54.17 ± 0.032% for plant extract and BHT, respectively. The concentrations that inhibit the hydrogen peroxide by 50 percent were determined as 34.32 ± 0.052 µg/mL in plant extract and 23.57 ± 0.29 µg/mL for BHT. It has been determined that the plant is less efficient than BHT in scavenging the hydroxyl peroxide.

Several hemolytic agents are known that induce hemolysis in animal models. One of these agents is phenylhydrazine (PHZ), a hydrazine derivative. The PHZ hemolytic action pathway has been linked to its association with RBCs. This reaction creates hydrogen peroxide, which produces oxidized compounds and hydrazine free radicals, eliminating the hemoglobin pigment. PHZ also causes the development of ROS, lipid peroxidation, and protein oxidation due to the plasma membrane's reaction (Allahmoradi *et al*., 2019).

In the present study, the anti-hemolytic activity of the methanol extract of *L. rantonnetii* plant was determined. The highest haemolysis value of the plant extract's phenylhydrazine was determined as 67.18 ± 7.33% and 50.77 ± 0.58% for trolox. The concentrations that inhibit the phenylhydrazine haemolysis by 50 percent were determined as 191.13 ± 52.51 µg/mL in plant and 16.17± 6.22 µg/mL for trolox. *L. rantonnetii* plant and trolox showed a differential pattern of anti-hemolytic activity. Their IC_{50} results indicate that the anti-hemolytic action of plant methanol extracts weaker than trolox.

The analysis result was showed that the plant is very rich in mineral content and contains high amounts of magnesium and phosphorus. The overall phenol and flavonoid composition of the plant was noted, and its antioxidant potential was also at a reasonable level. Moreover, it was concluded that the plant is effective in the inhibition of ROS, scavenging ABTS and DPPH radicals, which are stable free radicals, hydrogen peroxide and has a good activity of antioxidants and antiradical through hindering the hydroxyl radical, which in biological systems is the most responsive species. In conclusion, the *L. rantonnetii* plant has very high total phenolic, flavonoid and vitamin E, A, C content, mineral content, antioxidant, and antiradical activity. It may be considered for the treatment and prevention of many diseases caused by ROS. Moreover, reasonable amounts of total phenol and total flavonoids, and our research will consider future research as well. The results of the study revealed that *L. rantonnetii* can be used as an additive for food products in the various food sectors with appropriate antioxidant activities.

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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

Abdalla Ali Amin: Plant collection, Laboratory analysis, and Writing in the study. **Suat Ekin:** Investigation, Designing and Writing. **Ahmet Bakir:** Laboratory analysis. **Damla Yildiz:** Laboratory analysis.

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Research Article

Molecular Docking, Antibacterial and Antioxidant Activities of Compounds Isolated from Ethiopian Plants

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Abstract: This study evaluated the antibacterial and antioxidant activities of the constituents of *L. tomentosa* and *S. longipedunculata*. The *in-silico* molecular docking analysis of the isolated compounds was also reported herein for the first time. The GC-MS analysis of the essential oil of *L. tomentosa* led to the identification of eleven components with 2,5-dimethoxy-*p*-cymene identified as the principal constituent (59.39%). Lauric acid (**1**), *β*-stigmasterol (**2**), chrysophanol (**3**), and emodin (**4**) were isolated from *L. tomentosa* using silica gel column chromatography. Likewise, 9H-xanthene-3,5-diol (**5**), 1,7-dihydroxy-4-methoxyxanthone (**6**), and oleic acid (**7**) were isolated from *S. longipedunculata*. The structures of the isolated compounds were elucidated using UV-Vis, IR, and NMR spectroscopic methods. Compounds **3** and **4** are new to the genus Laggera, while **5** and **6** are new to the species *S. longipedunculata*. Compounds **3-6** inhibited DPPH radical by 86, 92, 88, and 90%, respectively. Compounds **5** and **6** inhibited 79.2 and 81.9% peroxide formation, respectively. The antioxidant activities displayed by compounds **4-6** suggest their use as a natural antioxidant. Compounds **4** and **6** inhibited the growth of bacteria by 18.00±0.10 and 16.06±0.22 mm, respectively. Compounds **3**, **4**, and **6** showed binding affinities of −10.4, −10.4, and −9.9 kcal/mol against *Staphylococcus aureus* DNA Gyrase, respectively, while **4** showed −10.4 kcal/mol against human topoisomerase IIβ. Therefore, the present study results showed that emodin and 1,7-dihydroxy-4-methoxyxanthone might be considered lead compounds for further development as antibacterial and anti-cancer agents. The findings also substantiate the traditional use of these plants against bacteria.

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1. INTRODUCTION

Laggera tomentosa (Asteraceae) is a perennial plant endemic to Ethiopia growing in Shewa, Arsi, Wollo, Gonder and Gojjam (Asfaw, Storesund, Aasen, & Skattebol, 2003). The plant is traditionally used in Ethiopia against tooth-ache, stomach-ache, swellings, ringworm, and infections. The compounds 2,4,4-trimethylbicyclo[3.1.1]hept-2-en-6-one, 2,7,7-

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trimethylbicyclo[3.1.1]hept-2-en-6-one, (2E,4E)-3,7-dimethylocta-2,4,6-trienal, (2Z,4E)-3,7-dimethylocta-2,4,6-trienal, 1,4-dimethoxy-5-methyl-2-(1-methylethyl)benzene have been reported from the essential oil of the leaves of *L. tomentosa* (Asfaw, Storesund, Aasen, & Skattebol, 2003). Furthermore, the leaves were reported to be rich in flavonoids including 4-*O*-acetylcuauthemone-3-*O*-(2'-hydroxy-2'-methyl-3'-acetoxybutyrate), 3-*O*-(3'-acetoxy-2'-hydroxy-2'-methylbutyryl)cuauthemone, 4-*O*-acetylcuauthemone-3-*O*-angelate, 3',5,6-trihydroxy-3,4',7-trimethoxyflavone, 3',4',5,7-tetrahydroxy-3,6-dimethoxyflavone, 4',5,7-trihydroxy-3',3,6-trimethoxyflavone, 3',5,7-trihydroxy-3,4',6-trimethoxyflavone (Kibrom, Dibaba, & Nigist, 2010).

Securidaca longipedunculata (Polygalaceae) is a popular hedge plant in Ethiopia traditionally used for washing clothes because of its foam-forming ability in the water. The plant is also used to treat arthritis, wounds, cough, venereal diseases, tuberculosis, and skin diseases (Debella *et al*., 2000) (Dibwe *et al*., 2013). Previous pharmacological reporting has shown that the extracts exhibit anti-inflammatory activity (Akpemi *et al*., 2013). Chemically, *S. longipedunculata* was reported to have quercetin-3-*O*-D-xyloside (Debella *et al*., 2000), benzyl-6-methoxybenzoate, cinnamic acid, caffeic acid, cinapic acid, *p*-coumaric acid and *β*-sitosterol (Mongalo *et al*., 2015). Though these plants are traditionally used to treat a wide array of diseases, there is no report on the isolation of compounds responsible for the antibacterial and antioxidant activities of the extracts of *L. tomentosa* and *S. longipedunculata*. Therefore, this paper presents the study results on the chemical constituents, antibacterial and radical scavenging activities of the root extracts of *L. tomentosa*, and stem barks of *S. longipedunculata*. Also incorporated herein are the reports on the *in-silico* molecular docking, ADMET, and DFT analysis of compounds isolated from *L. tomentosa* and *S. longipedunculata*.

2. MATERIAL and METHODS

2.1. Plant Material

L. tomentosa and *S. longipedunculata* were collected in November 2018 from Sebeta and Menesibu, Ethiopia, respectively. A botanist identified the plants, and voucher specimens GW003 for *L. tomentosa* and M002 for *S. longipedunculata* were deposited at the National Herbarium of Addis Ababa University, Ethiopia.

2.2. Extraction and Isolation

2.2.1. *Roots of laggera tomentosa*

The root of *L. tomentosa* (300 g) was successively extracted with each 1.2 L of *n*-hexane, EtOAc, and MeOH using maceration for 72 hrs. Each extract was filtered and concentrated by a rotary evaporator at 40^{o}C to afford 3.22, 5.06, and 6.88 g, respectively. The EtOAc extract (4.06 g) was fractionated over silica gel (160 g) column chromatography using *n*-hexane: EtOAc: MeOH as eluent to give ten fractions, each 300 mL. Fractions 1, 2, 3, 4 and 5 were eluted using *n*-hexane: EtOAc 9:1, 7:3, 3:2, 3:7 and 1:9, respectively. Fraction 6 was eluted with 100% EtOAc. Fractions 7, 8, 9 and 10 were eluted with EtOAc: MeOH in 9:1, 8:2, 7:3 and 1:1, respectively. Fractions eluted with *n*-hexane: EtOAc (7:3) after silica gel column chromatography gave compound **1** (19 mg) while compounds **2** (21 mg) and **3** (9 mg) were isolated from the fraction eluted with *n*-hexane: EtOAc (3:7). Fraction 7 was fractionated over silica gel column chromatography using *n*-hexane: EtOAc (1:9) to afford six fractions, 25 mL each. Sub-fraction 3 was found to be compound **4** (13 mg).

2.2.2. *Stem barks of securidaca longipedunculata*

The stem bark of *S. longipedunculata* (500 g) was successively extracted using maceration using *n*-hexane, EtOAc, and MeOH, each 2.5 L for 72 hrs. A rotary evaporator filtered and concentrated the extracts at 40^{o}C to give 2.6, 5, and 51 g. The EtOAc extract (5 g) was subjected

to silica gel (150 g) column chromatography using the increasing polarity of EtOAc in *n*-hexane as eluent to afford 40 fractions, each 100 mL. The First 1-5 were eluted with *n*-hexane. The next three fractions were eluted with *n*-hexane: EtOAc (9:1). Fractions 9-11 were eluted with *n*-hexane:EtOAc (4:1). Fraction 12-14, Fraction 15-17, Fraction 18-21, Fraction 22-24, Fraction 25-27, Fraction 28-30, Fraction 31-34 were collected using *n*-hexane:EtOAc (7:3), *n*-hexane:EtOAc (6:4), *n*-hexane:EtOAc (1:1), *n*-hexane:EtOAc (4:6), *n*-hexane:EtOAc (3:7), *n*-hexane:EtOAc (1:4) and *n*-hexane:EtOAc (1:9), respectively. The last six fractions were collected using EtOAc. Fraction 15-18, eluted with *n*-hexane: EtOAc (3:2), was identified as compound **5** (17 mg) as yellow crystals. Fraction 18-21, eluted with *n*-hexane: EtOAc (1:1), was identified as compound **6** (8 mg). Fractions eluted with *n*-hexane: EtOAc (3:7) after silica gel column chromatography gave compound **7** (7 mg) as a white solid.

2.3. GC-MS analysis of *n*-hexane extract of the roots of *L. Tomentosa*

The essential oil obtained from the *n*-hexane extract of *L. tomentosa* was analyzed using Agilent Technologies 7820A GC system with Agilent technologies 5977E mass selective detector, USA.

2.4. Antioxidant Activity

2.4.1. *Radical scavenging activity*

The radical scavenging activity of the samples was evaluated using DPPH as described in the previously reported protocol (Brand-Williams, Cuvelier, & Berset, 1995). The standard solution (500 μg/mL) was prepared by dissolving each sample in MeOH. Each solution was serially diluted in 0.1 mM DPPH in MeOH to give 100, 50, 25, and 12.5 μg/mL. After an incubation period of 30 minutes at 37°C, the absorbance of the sample was measured at 517 nm using a UV-Vis spectrophotometer (Ghasemi *et al*., 2009). This measurement was repeated for ascorbic acid. The experiments were performed in triplicates and reported as M±SD. The % radical scavenging activity was then calculated with the following formula (Qusti *et al*., 2010):

$$\% \text{ Inhibition} = \frac{(A_{control} - A_{extract})}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of DPPH solution and $A_{extract}$ is the absorbance of the test sample (DPPH solution plus sample)

2.4.2. *Ferric thiocyanate method*

The ferric thiocyanate method was used to assess the anti-lipid peroxidation potential of the samples following the previously reported procedure (Nagatsu, 2004). Each sample (0.1 mg), linoleic acid (100 µL), EtOH (5 mL), and phosphate buffer (5 mL, 0.05 M, pH = 7) in water were added into a vial and then incubated for 24 h at 40°C. An aliquot (0.1 mL) was taken and added into a vial containing 75% aqueous ethanol (7 mL), 30% of NH_4SCN (0.15 mL) and 0.15 mL of 0.02M $FeCl_2$ in 3.5% HCl. The samples were then subjected to a UV-Vis spectrophotometer to measure the absorbance at 500 nm. This measurement was repeated for the blank and ascorbic acid. The % inhibition is calculated using the following formula (Gülçin *et al*., 2010).

$$Percentage\ inhibition = 100 - \left(\frac{As}{Ab} x100\right)\%$$

Where As is absorbance of the sample and Ab is absorbance of the blank.

2.5. Antibacterial Activities

The samples were evaluated for their *in-vitro* antibacterial activity using the disc diffusion method against *E. coli* (ATCC25922), *P. mirabilis* (ATCC 35659), *K. pneumoniae*

(ATCC700603), *S. typhi* (ATCC1331), *S. aureus*, (ATCC25923) and *B. subtilis* (ATCC6633) (Ołdak *et al*., 2017). The bacterial species were transferred from the stock cultures, and microbial suspensions were prepared in a nutrient broth for 24 hrs at 37°C until the turbidity of bacterial suspensions was achieved to 1.5×10^8 CFU mL^{-1} by comparison with the 0.5 McFarland standard. The assay was carried out by swabbing each test strain on the Mueller-Hinton agar plate using the 1/10 dilution of the microbial suspension. Sterile paper discs (Whatman No.1 filter paper) impregnated with 0.5 mg/mL and 1 mg/mL samples in DMSO were placed onto the surface of the agar plate at equal distances from each other. Chloramphenicol was used as a positive control. After overnight incubation at 37°C, zones of inhibition were measured. The samples were analyzed in triplicates.

2.6. *In-silico* Molecular Docking Analysis of the Isolated Compounds

The structures of the isolated compounds were drawn using ChemDraw 16.0. The molecules were treated quantum mechanically using the Gaussian 09 program suite at the Becke-3-Lee-YangPar (B3LYP) level combined with the standard 6-31G (d,p) basis set. All the parameters were set to get a stable structure with minimum energy. Each molecule's 3D coordinates (PDB) were obtained through the optimized structure. The crystal structures of *S. aureus* DNA Gyrase (PDB ID 2XCT) and human topoisomerase IIβ (PDB ID 3QX3) were downloaded from the protein data bank. The protein preparation was done using the reported standard protocol using AutoDock 4.2.6 (MGL tools 1.5.6). The AutoDock Vina searched for the best-docked conformation between isolated compounds and protein. A maximum of nine conformers were considered for each ligand in the docking process. The conformations with the least free binding energy were selected for analyzing the interactions between the target receptor and ligands by discovery studio visualizer and PyMOL. The molecular docking studies were carried out using AutoDock Tools (ADT) (Trott & Olson, 2010).

2.7. *In-silico* Pharmacokinetics and Toxicity of the Isolated Compounds

The structures of the isolated compounds were converted to their canonical simplified molecular-input line-entry system (SMILES) and submitted to the SwissADME tool to estimate *in-silico* pharmacokinetic parameters. The information about the number of hydrogen donors, hydrogen acceptors, rotatable bonds, and total polar surface area of the isolated compounds was obtained from the SwissADME predictor. The ligands were also subjected to Lipinski *et al*., screened using SwissADME and PreADMET predictors. The ligands' organ toxicities and toxicological endpoints and their LD_{50} were predicted using Pro Tox II and OSIRIS Property Explorer (Banerjee *et al*., 2018) (Garg, Tadesse, & Eswaramoorthy, 2021). The analyses of the compounds were compared with that of Doxycycline and Abiraterone standard drugs.

2.8. DFT Study

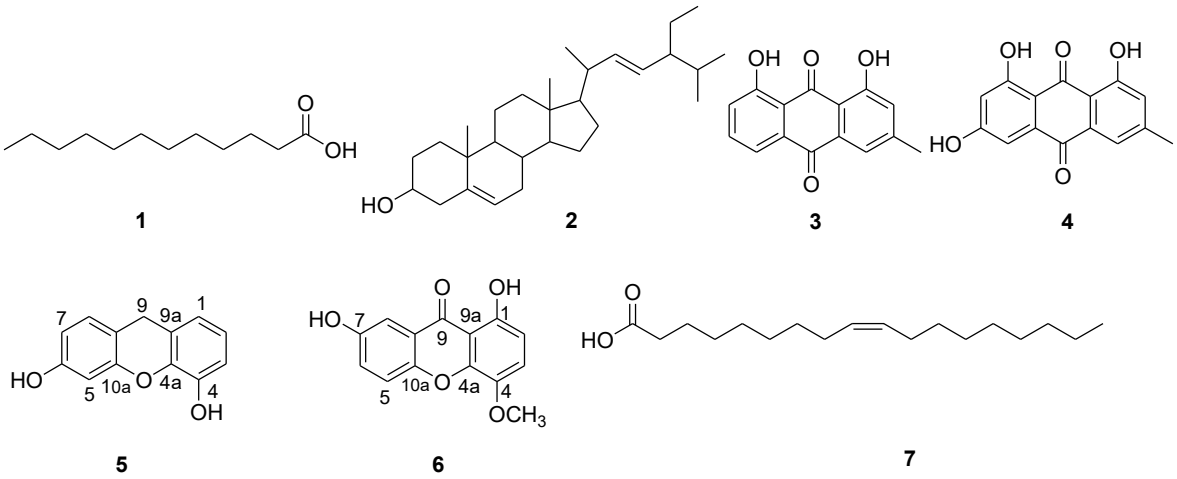
The isolated compounds were treated quantum mechanically with the DFT method using the Gaussian 09 program suite at the Becke-3-Lee-YangPar (B3LYP) level combined with the standard 6-31G (d,p) basis set without any symmetrical constraints. The DFT analysis of isolated compounds was performed using Gaussian 09 and visualized through Gauss view 6.0. The parameters were set to obtain a stable structure with minimum energy during the optimization procedure. The minimum global energy of the title compound was determined from the structure optimization procedure. The molecular electrostatic potential map and energies of the compounds were obtained from the optimized geometry. Koopman's approximation was used to estimate the HOMO-LUMO energy gap and related reactive parameters (electronegativity, chemical potential, hardness, softness, electrophilicity) (Abu-Melha, 2018; Vujovic *et al*., 2020).

3. RESULTS and DISCUSSION

3.1. Characterization

Seven compounds (Figure 1) were isolated and characterized in the present work. The structures of the compounds were established using various spectroscopic methods, including UV-Vis, IR, and NMR.

Figure 1. Chemical structures of isolated compounds.



Compound **1**, obtained as a solid (19 mg), showed a spot on TLC at Rf. 0.75 with n-hexane: EtOAc (9:1) as a mobile phase. The IR and NMR spectral data of compound **1** agreed with the data reported for lauric acid (Yamashita *et al*., 2015).

Compound **2** (21 mg) was obtained as white crystals. The UV-Vis spectrum (ethanol) showed absorption maxima at 259 nm, indicating the presence of non-conjugated olefinic (C=C) groups. The IR (KBr) spectrum revealed the presence of O-H and C=C stretching at 3360 and 1638 cm^{-1}, respectively. The NMR spectral data of compound **2** were in good agreement with data reported for *β*-stigmasterol (Figure 1) (Chaturvedula & Prakash, 2012), which has been reported as a remedy for reducing the risk of heart disease and blood cholesterol. It also possesses potent laxative, antioxidant, antibacterial, hypoglycemic, and thyroid inhibiting properties (Panda *et al*., 2009).

Compound **3** (9 mg) was obtained as a yellow amorphous powder. The UV-Vis spectrum showed characteristic absorption bands for anthraquinone at λ_{max} 266, 288, and 435 nm (Coopoosamy & Magwa, 2006). The IR (KBr) spectrum displayed absorption band at 3433 cm^{-1} is due to O-H stretching. The NMR spectral data of **3** (Table 1) were compared with the literature reported for 1,8-dihydroxy-3-methylanthracene-9,10-dione (chrysophanol) and found in good agreement (Coopoosamy, & Magwa, 2006). So far, this compound has not been reported from the genus *Laggera*. Chrysophanol has been reported from *Aloe excelsa*, *Rheum emodi*, *Rhamnus prinoides*, *Rumex abyssinicus*, *Rumex japonicus*, and *Cassia tora* (Coopoosamy, & Magwa, 2006; Guo *et al*., 2011; Tamano & Koketsu, 1982).

Table 1. NMR spectral data of compound **3** and data reported for chrysophanol.

Carbon No	Compound 3		Lit. for chrysophanol
	^{1}H-NMR Data	^{13}C-NMR data	^{13}C-NMR data
1	–	162.4	162.4
2	7.12 (br. *s*, 1H)	124.5	124.5
3	–	149.3	149.3
4	7.67 (br. *s*, 1H)	119.9	119.9
5	7.83 (1H, *dd*, *J* = 1.2, 7.6 Hz)	136.9	136.9
6	7.69 (1H, *dd*, *J* = 8.4, 7.6 Hz)	124.4	124.4
7	7.30 (1H, *dd*, *J* = 1.2, 8.4 Hz)	121.4	121.4
8	–	162.7	162.7
9	–	192.5	192.5
10	–	182.0	181.9
11	–	133.6	133.6
12	–	115.9	115.7
13	–	113.7	113.4
14	–	133.3	133.3
15	2.48 (3H, s)	22.3	22.3

Chrysophanol has been reported as antimicrobial, anti-inflammatory, anti-mutagenic, and antidiabetic, inhibits poliovirus replication, and is active against HIV-1 protease (HIV-1 PR). The compound is also a potent photosensitizer (Rawat *et al*., 2013).

Compound **4** (13 mg) was obtained as an orange amorphous powder with an Rf. 0.55 in n-hexane:EtOAc (7:3) as a mobile phase. The UV-Vis spectrum (ethanol) displayed absorption bands at λ_{max} 250, 266, 289, and 436 nm characteristics of anthraquinone (Fekade, 2008). In the IR (KBr) spectrum, the absorption band at 3382 cm^{-1} is ascribed to O-H stretching. The NMR spectral analysis of compound **4** (Table 2) was in good agreement with the data reported for 1,6,8-trihydroxy-7-methylanthracene-9,10-dione, also known as emodin (Rawat *et al*., 2013) (Figure 1). Emodin has also not been reported from the genus but has been reported from *Senna occidentalis*, *Rheum palmatum*, *Rumex abyssinicus*, and the *Murrya koenigii*. The compound has been reported to possess antimicrobial, antiviral, anti-inflammatory, anti-ulcerogenic, immunosuppressive, and chemopreventive activities (Muto *et al*., 2007).

Table 2. NMR spectral data of compound **4** and data reported for emodin (Fekade, 2008).

Carbon No	Compound 4		Lit. for emodin
	^{1}H-NMR Data	^{13}C-NMR data	^{13}C-NMR data
1	–	162.3	162.4
2	7.12 (1H, m)	124.0	124.1
3	–	148.6	148.7
4	7.54 (1H, m)	120.5	120.6
5	7.23 (1H, *d*, *J* = 2.4 Hz)	108.8	108.8
6	–	165.5	165.5
7	6.65 (1H, *d*, *J* = 2.4 Hz)	107.9	107.9
8	–	165.3	165.4
9	–	190.7	190.9
10	–	181.2	181.3
11	–	135.6	135.4
12	–	109.5	109.6
13	–	113.5	113.6
14	–	133.3	133.4
15	2.46 (3H, s)	21.1	21.2

Compound **5** (17 mg) was obtained as a yellow crystal melting at 102°C. The IR spectrum showed an absorption band at 3394 cm^{-1} attributed to O-H stretching. The spectrum also displayed a band at 1610 cm^{-1} attributed to the C=C bond stretching of an aromatic ring. The ^{1}H NMR spectrum of compound **5** (Table 3) showed the presence of signals due to six protons on the aromatic ring. Three aromatic protons were observed at δ 7.54 (1H, d, J = 8.4 Hz), 6.89 (1H, d, J = 2.8 Hz), and 7.48 (1H, dd, J = 2.8, 8.4 Hz) were accounted to H-8, H-5, and H-7, respectively. The remaining at δ 7.26 (1H, dd, J = 2.8, 9.2 Hz), δ 7.41 (1H, dd, J = 7.2, 9.2 Hz), and δ 6.72 (1 H, d, J = 7.2 Hz) were due to the signals of H-3, H-2, and H-1, respectively. The spectrum also displayed a singlet signal at δ 3.48 (2H) diagnostic for the presence of methylene protons in the compound. The proton decoupled ^{13}C-NMR spectrum of compound **5** with the aid of DEPT-135 revealed the presence of 13 well-resolved carbon resonances, including one methylene, six methine, and six quaternary carbons. The signal due to methylene carbon is evident at δ 29.6. The remaining twelve signals were observed in the aromatic region, suggesting the presence of two non-equivalent aromatic rings in the structure of the compound. The signals in the aromatic ring were observed at δ 136.5 (C-8), 108.2 (C-7), 161.2 (C-6), 107.1 (C-5), 156.4 (C-10a), 128.1 (C-8a), 153.4 (C-4), 109.6 (C-3), 125.3 (C-2), 119.1 (C-1), 150.2 (C-4a) and at 128.6 (C-9a). Signals due to quaternary carbons were apparent at δ 161.2 (C-6), 156.4 (C-10a), 128.1 (C-8a), 153.4 (C-4), 150.2 (C-4a) and at 128.6 (C-9a). The most downfield signals at δ 161.2, 156.4, 153.4, and 150.2 are signals due to oxygenated aromatic carbons. The below spectral data led to the identification of compound **5** as 9H-xanthene-3, 5-diol, whose structure is depicted in Figure 1.

Table 3. ^{1}H NMR, ^{13}C NMR and DEPT-135 spectral data of compound **5**.

Position	^{1}H NMR δ (ppm)	^{13}C NMR δ (ppm)	DEPT-135
1	6.72 (1H, *d*, *J* = 7.2 Hz)	119.1	CH
2	7.41 (1H, *dd*, *J* = 7.2 and 9.2 Hz)	125.3	CH
3	7.26 (1H, dd, J = 2.8 and 9.2 Hz)	109.6	CH
4	–	153.4	Q
4a	–	150.2	Q
5	6.89 (1 H, *d*, *J* = 2.8 Hz)	107.1	CH
6	–	161.2	Q
7	7.48 (1H, *dd*, *J* = 2.8 and 8.4 Hz)	108.2	CH
8	7.54 (1 H, *d*, *J* = 8.4 Hz)	136.5	CH
8a	–	128.1	Q
9	3.48 (2H, s)	29.6	CH_2
9a	–	128.6	Q
10a	–	150.2	Q

Compound **6** (8 mg) was obtained as a yellow crystal, melting at 236-237°C. The IR spectrum revealed an absorption band at 3286 cm^{-1}, illustrating the presence of O-H stretching in the compound. The absorption band at 1667 cm^{-1} is presumably due to a conjugated carbonyl group. The absorption band due to the C=C bond is evident at 1640 cm^{-1}. The ^{1}H-NMR spectrum of compound **6** (Table 4) showed the presence of five protons on the aromatic ring. Two proton ortho coupled signals with AB multiplicity pattern at δ 6.61 (1H, d, J = 8.8 Hz) and at δ 7.17 (1H, d, J = 8.8 Hz) are accounted for the C-H aromatic protons on C-2 and C-3, respectively. Three aromatic protons were observed at δ 7.24 (1H, dd, J = 2.8 and 9.2 Hz), δ 7.42 (1 H, d, J = 9.2 Hz), and δ 7.45 (1 H, d, J = 2.8 Hz) for H-6, H-5, and H-8, respectively. The ^{1}H-NMR spectrum also displayed a signal from the methoxy group at δ 3.88. The ^{13}C-NMR spectrum of compound **6** in combination with DEPT-135 suggested the presence of 12

aromatic carbons, one methoxy carbon, and one C=O carbonyl carbon. The carbon signals resonating at δ 154.0 (C-1), 107.9 (C-2) 119.8 (C-3), 140.2 (C-4), 145.9 (C-4a) and 108.9 (C-9a) suggests the presence of six aromatic carbons. The spectrum also displayed other carbon resonances in the aromatic region at δ 119.3 (C-5), 125.3 (C-6), 153.8 (C-7), 108.1 (C-8), 120.0 (C-8a) and 150.1 (C-9a). The aromatic signals that bear hydroxyl groups were apparent at δ 153.8 and 154.0. The signals at δ 145.9 and 150.1 are accounted for the two quaternary carbons that bonded to the oxygen atom, hence assigned to C-10a and C-4a, respectively. The OH group must be situated para to the methoxy group to fit with that chemical shift (140.2 ppm), as reported in Yan (2001). The most downfield signal at δ 182.2 is characteristic of conjugated carbonyl (C-9). The signals which were not detected in the DEPT-135 NMR spectrum of compound **6**, including δ 108.9, 120.0, 140.2, 145.9, 150.1, 153.8, 154.0, and 182.2, were due to quaternary carbons. The signal due to the methoxy group was detected at δ 57.2.

Table 4. ^{1}H NMR, ^{13}C NMR, and DEPT-135 spectral data of compound **6**.

Position	^{1}H NMR δ (ppm)	^{13}C NMR δ (ppm)	DEPT 135 NMR
1	_	154.0	Q
2	6.61 (*1H, d, J* = 8.8 Hz)	107.9	(CH)
3	7.17 (1 *H, d J* = 8.8 Hz)	119.8	(CH)
4	_	140.2	Q
4a	_	145.9	Q
5	7.42 (1H, *d*, *J* = 9.2 Hz)	119.1	(CH)
6	7.24 (1H, *dd*, *J* = 2.8 and 9.2 Hz)	125.3	(CH)
7	_	153.8	Q
8	7.46 (1H, *d*, *J* = 2.8 Hz)	108.1	(CH)
8a	_	120.0	Q
9	_	182.2	Q
9a	_	108.9	Q
10a	_	150.1	Q
−OCH_3	3.88 (3H, s)	57.2	(CH_3)

With the above spectral data, compound **6** is identified as 1,7-dihydroxy-4-methoxyxanthone (Figure 1). Compound **7** was obtained as a white powder. The NMR spectral data and melting point of compound **7** is identical to oleic acid (Baker *et al*., 1995).

3.2. GC-MS analysis of the *n*-hexane extract of the roots of *L. tomentosa*

The essential oil of the roots of *L. tomentosa* has led to the identification of eleven major chemical constituents. It was found that the major component of the n-hexane extract of the root of *L. tomentosa* was 2,5-dimethoxy-*p*-cymene which constituted 59.39% followed by 1-isopropyl-2-methoxy-4-methylbenzene (9.54%), 2-(methylamino)-1-phenylethanol (7.56%), tetradecyl 2-(N-methylisobutyramido)acetate (6.54%), methyl palmitate (5.74%), 1-guanidinosuccinimide (3.77%), propanamide (1.96%), α-humulene (1.86%), N-methylpent-4-en-1-amine (1.29%), 2-fluoroamphetamine (1.20%) and sec-butylamine (1.15%). 2,5-Dimethoxy-*p*-cymene was also reported as one of the constituents from the essential oil of the aerial parts of *L. tomentosa* grown only in Ethiopia, which agrees well with the present work. However, the content of 2,5-dimethoxy-*p*-cymene reported from *L. alata* (44.2%) grown in Nigeria and *L. pterodonta* (30.5%) grown in the Benin Republic are inferior to the percent reported in this work (Omoregie, Okwute, & Koma, 2014; Verma *et al*., 2011; Hakim *et al*.,

2008). This compound was also reported from various plants' essential oils obtained from the family Asteraceae (Owolabi, Lajide, Villanueva, & Setzer, 2010). The presence of volatile components like 2,5-dimethoxy-*p*-cymene and 1-isopropyl-2-methoxy-4-methylbenzene suggests that the oil may be useful in aromatherapy, pharmaceuticals, and confectionery industries as fragrance or additives (Martin *et al*., 2000). The essential oils which contain this compound as a major component were reported to have antifungal (Hakim *et al*., 2008), antibacterial (Joshi, 2013), and insecticidal (Owolabi *et al*., 2010) properties. Therefore, the presence of 2,5-dimethoxy-*p*-cymene as a major constituent in the roots of *L. tomentosa* is one positive attribute of this plant.

3.3. Antioxidant Activities

3.3.1. *DPPH radical scavenging activity*

DPPH radical scavenging assay is a simple method for finding antioxidants by recording the absorbance at 517 nm (Brand-Williams, Cuvelier, & Berset, 1995). The extracts and isolated compounds from the roots of *L. tomentosa* were evaluated for their radical scavenging activities, and the result is presented in Table 5.

Table 5. DPPH Radical Scavenging Activities of the Extracts and Isolated Compounds (100 µg/mL).

Roots of *L. tomentosa*			Stem barks of *S. longipedunculata*		
Samples	% Inhibition	IC_{50} value	Samples	% Inhibition	IC_{50} value
n-hexane extract	55.8±0.3	92.0	*n*-hexane extract	42.0±0.8	218.0
EtOAc extract	84.3±0.2	9.4	EtOAc extract	89.1±0.7	7.4
MeOH extract	72.5±0.6	29.0	MeOH extract	91.0±0.3	6.4
2	37.8±0.4	1150.0	**5**	88.0±0.6	8.4
3	86.0±0.6	6.2	**6**	90.2±0.5	4.2
4	92.0±0.1	3.8	**Ascorbic acid**	97.0±009	3.38

Samples were reported as Mean ± SEM; IC_{50} values were reported in µg/mL

As shown in Table 5, the *n*-hexane extract of the roots of *L. tomentosa* inhibited the DPPH radical by 55.8%. The percentage inhibitions of the EtOAc and MeOH extracts and compounds **2**, **3**, and **4** are 84.3, 72.5, 37.8, 86.0, and 92%, respectively. The IC_{50} values were 92, 9.4, 29, 1150, 6.2, and 3.8 µg/mL for hexane, EtOAc, and MeOH extract, and compounds **2**, **3**, and **4** isolated from the roots of *L. tomentosa*, respectively. The corresponding result of 3.38 µg/mL was recorded for ascorbic acid. As clearly revealed from Table 1, compounds **3**, **4**, and the EtOAc extract displayed comparable radical scavenging activities with the standard. This activity is likely due to hydroxyl groups in anthraquinones **3** and **4**. The behavior agrees with the IC_{50} values of compounds **3** (6.2 µg/mL) and **4** (3.8 µg/mL). Therefore, the results showed that the roots of *L. tomentosa* can serve as a source of radical scavenging compounds and hence can be utilized as a natural antioxidant.

The hexane, EtOAc, and MeOH extract of the stem barks of *S. longipedunculata* inhibited DPPH radical by 42, 89, and 91% (Table 5). This percentage indicates that the MeOH and EtOAc extract inhibited the DPPH radicals significantly compared with the hexane extract. This activity might be due to flavonoids and was confirmed using Salkowski Test in the EtOAc and MeOH extracts compared to the *n*-hexane extract. This result is also evident from the low IC_{50} of the EtOAc (7.42 µg/mL) and MeOH extract (6.48 µg/mL). At the same concentration, ascorbic acid scavenged the DPPH radical by 97.0% with an IC_{50} value of 3.38. Compounds **5** and **6** inhibited DPPH radical by 88 and 90.2%, respectively. The result is almost close to ascorbic acid used as a positive control. Therefore, the observed high free radical scavenging

activity of the EtOAc and MeOH extract may be accounted to the presence of compounds **5** and **6**, isolated in this study from the stem bark of *S. longipedunculata*.

3.3.2. *Ferric thiocyanate method*

The degree of lipid peroxidation, which was evaluated using the ferric thiocyanate method, can measure the antioxidant potential of compounds or extracts. Table 6 is the result of the anti-lipid peroxide formation of the constituents of *L. tomentosa* and *S. longipedunculata*.

Table 6. Anti-lipid peroxidation activities of constituents of *L. tomentosa* and *S. longipedunculata.*

Stem bark of *S. longipedunculata*		Roots of *L. tomentosa*	
Samples	% Inhibition	Samples	% Inhibition
n-hexane extract	47.2±0.4	*n*-hexane extract	35.2±0.2
EtOAc extract	70.8±1.0	EtOAc extract	78.5±0.8
MeOH extract	76.4±0.7	MeOH extract	74.1±1.1
5	79.2±0.1	Ascorbic acid	84.7±0.4
6	81.9±0.3		

Samples were reported as Mean ± SEM; Ascorbic acid was used as positive control

As depicted in Table 6, the anti-lipid peroxidation activity of the *n*-hexane extract is inferior to the other extracts and isolated compounds. On the other hand, the EtOAc and MeOH extracts of *S. longipedunculata* inhibited peroxide formation by 70.8 and 76.4%, respectively, demonstrating their potential in preventing the formation of lipid peroxides. The results turned out to be comparable with ascorbic acid, which inhibits the formation of lipid peroxides by 84.7%. Likewise, compounds **5** and **6** were shown to have a high ability to inhibit peroxide formation, which is comparable with ascorbic acid. The anti-lipid peroxidation activity of the *n*-hexane, EtOAc, and MeOH extracts of the roots of *L. tomentosa* displayed % inhibition of 35.2, 78.5, and 74.1%, respectively. The result demonstrated by EtOAc extract is close to ascorbic acid.

3.4. Antibacterial Activity

The extracts and isolated compounds from *L. tomentosa* were assayed *in-vitro* against *S. aureus*, *E. coli*, *P. mirabilis*, and *K. pneumoniae* (Table 7). The MeOH and *n*-hexane extracts were significantly active against *S. aureus* and *E. coli* at 1.0 mg/mL, displaying an inhibition zone of 11-13 mm. The EtOAc extract had an IZ of 12 mm against *E. coli* than other strains used in this study. At 0.5 mg/mL, EtOAc and MeOH extracts demonstrated moderate antibacterial effects (7-10 mm) against all the tested bacteria, indicating that the samples inhibit the pathogens in a dose-dependent manner. The activity of EtOAc and MeOH extract (at 1 mg/mL concentration) of the roots of *L. tomentosa* against the Gram-negative [*K. pneumoniae* (10 mm, 9 mm), *E. coli* (12 mm, 12 mm), and *P. mirabilis* (11 mm, 9 mm)] respectively is impressive because Gram-negative bacteria tend to have higher intrinsic resistance to most antimicrobial agents (Ndukwe, Okeke, Lamikanra, Adesina, & Aboderin, 2005). Compounds **1**, **2**, and **3** isolated from this plant were shown to have a high zone of inhibition with diameters of (10 mm, 8 mm, and 11 mm), (12 mm, 11 mm, and 10 mm), and (13 mm, 11 mm and 9 mm) against *S. aureus*, *K. pneumoniae*, and *E. coli*, respectively. However, the activity displayed by these compounds is inferior to compound **4**. Hence, fractionations of the active EtOAc extract of *L. tomentosa* over silica gel column chromatography traced the activity to emodin (4) with an inhibition zone of 18 mm against *S. aureus*. This agreement is consistent with the solid antimicrobial activities reported for compound **4** from different sources (Muto *et al*., 2007). Hence, the result obtained in the present study supports the traditional use of *L. tomentosa* against diseases caused by bacteria.

Table 7. Zone of Bacterial Growth Inhibition (mm) of Extracts and Isolated Compounds.

Samples	Roots of *L. tomentosa*				
	Conc. in mg/mL	Zones of inhibition in mm			
		S. aureus	*K. pneumoniae*	*E. coli*	*P. mirabilis*
n-hexane extract	0.5	NI	7.10±0.40	NI	NI
	1.0	12.00±0.20	10.21±0.30	11.01±0.10	NI
EtOAc extract	0.5	9.01±0.41	7.10±0.21	10.01±0.30	8.10±0.40
	1.0	19.01±0.31	10.00±0.30	12.10±0.20	11.10±0.40
MeOH extract	0.5	8.10±0.30	9.01±0.20	9.20±0.21	8.10±0.20
	1.0	13.00±0.20	7.00±0.21	12.20±0.40	9.10±0.20
1	0.5	8.20±0.30	7.10±0.20	8.10±0.30	NI
	1.0	10.21±0.20	8.21±0.20	11.20±0.21	NI
2	0.5	8.40±0.30	10.00±0.40	9.00±0.20	8.11±0.30
	1.0	12.00±0.20	11.10±0.41	10.10±0.30	10.11±0.11
3	0.5	7.10±0.10	8.10±0.10	7.10±0.10	NI
	1.0	13.00±0.20	11.21±0.30	9.10±0.10	NI
4	0.5	8.00±0.20	10.21±0.10	7.20±0.21	NI
	1.0	18.00±0.10	12.10±0.30	8.20±0.20	NI
Chloramphenicol	1.0	18.00±0.10	18.10±0.10	18.20±0.10	18.10±0.11
Stem barks of S. longipedunculata					
Samples	Conc. in mg/mL	Zones of inhibition in mm			
		E. coli	*S. aureus*	*S. typhi*	*B. subtilis*
n-hexane extract	0.5	8.10±0.22	10.04±0.23	6.01±0.21	NI
	1.0	12.10±0.02	14.04±0.07	7.08±0.31	NI
EtOAc extract	0.5	7.00.09±0.02	11.00±012	9.09±0.21	NI
	1.0	11.08±0.04	15.03±018	12.09±0.42	NI
MeOH extract	0.5	7.11±0.03	9.00±0.33	7.20±0.21	NI
	1.0	11.21±0.07	12.00±0.28	8.00±0.31	NI
5	0.5	8.11±0.10	12.00±0.09	10.10±0.21	NI
	1.0	11.31±0.10	15.09±0.09	15.12±0.31	6.00±0.03
6	0.5	9.21±0.21	11.26±0.11	12.11±0.07	6.40±0.11
	1.0	14.19±0.14	16.06±0.22	15.28±0.09	8.20±0.21
Chloramphenicol	1.0	18.20±0.10	18.10±0.10	19.20±0.30	21.10±0.19

NI: no inhibition; Results are mean ± SD of triplicate experiments

Likewise, the extracts and isolated compounds from *S. longipedunculata* were assessed for their *in-vitro* antibacterial activity against *E. coli*, *S. aureus*, *S. typhi*, and *B. subtilis*. The results are summarized in Table 7. The *n*-hexane, EtOAc, and MeOH extracts of *S. longipedunculata* showed inhibition zones of 14.04±0.07, 15.03±018, and 12.00±0.28 against *S. aureus* at 1 mg/mL. On the other hand, the EtOAc extract of *S. longipedunculata* showed an inhibition zone of 12.09±0.42 against *S. typhi*, while the *n*-hexane and MeOH extract were turned out to be inactive. This result was in close agreement with the one reported in the literature on the same bacterial strain (Declercq *et al*., 2001). None of the extracts and isolated compounds demonstrated activity against *B. subtilis*. Fractionation of the active EtOAc extract has led to the isolation of two xanthones that displayed activity against *S. aureus*, *E. coli*, and *S. typhi*. Compound **5** exhibited inhibition zones of about 11.31±0.10, 15.09±0.09, and 8.00±0.31

against *E. coli*, *S. aureus*, and *S. typhi*, respectively. Likewise, compound **6** displayed an inhibition zone of 14.19±0.14, 16.06±0.22, and 15.28±0.09, respectively. As clearly observed from Table 3, compounds **5** and **6** displayed similar activity with the standard drug against *S. aureus* with an inhibition zone of 15.09±0.09 and 16.06±0.22 mm, respectively. Therefore, the activity shown by the extract could be accounted to the presence of the two xanthones in the extract.

3.5. Molecular Docking Studies

Molecular docking studies are generally employed to investigate the binding energy and to further validate the molecular mechanisms for ligands at the active site of a protein. All the isolated compounds were subjected to molecular docking studied against selected proteins viz. *S. aureus* DNA Gyrase (PDB ID 2XCT) and human topoisomerase IIβ with DNA (PDB ID 3QX3) using Auto-dock Vina (Trott & Olson, 2010) to find out the binding mode.

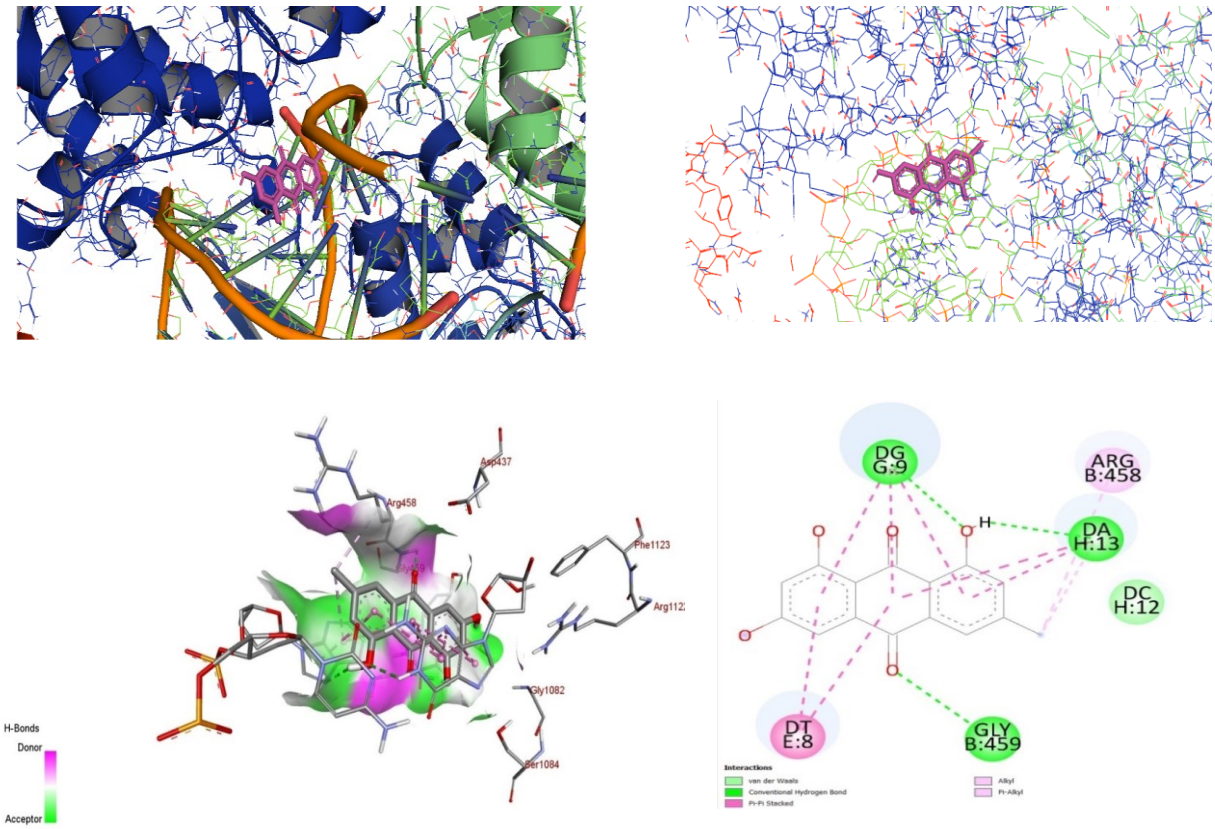
S. aureus DNA Gyrase, an enzyme belonging to a member of bacterial topoisomerase, controls the topology of DNA during transcription, replication, and recombination by introducing short breaks to both DNA strands. Hence, the bacterial gyrase is paramount for bacterial survival and, therefore, necessary to disrupt as an antibacterial drug target (El-Etrawy & Sherbiny, 2021). Therefore, in this study, the molecular docking analysis of the isolated compounds was carried out to investigate their binding pattern with bacterial gyrase. The results were compared with standard antibacterial agents ciprofloxacin and doxycycline. The isolated compounds were found to have minimum binding energy ranging from – 4.8 to – 10.4 kcal/mol (Table 8). Compounds **1** (Arg-458), **2** (Glu-1088), **3** (Asp-437, Gly-459), and **4** (Gly-459) have shown hydrogen bonding interaction with various amino acid residues, and few additional hydrogen-bonding interactions are also shown with bacterial DNA (Table 8). Compounds **5-6** showed the residual amino acids interactions with Ala-47, Glu-50, Gly-77, Ile-78, Pro-79, Ile-94, Thr-165 (Hydrophobic), and Asp-73, Arg-76, Asn-46 (Hydrogen bond) similar to ciprofloxacin and doxycycline within the binding site of the protein. Compound 5 forms hydrogen bond interaction with Arg-76, Gly-77, and Thr-165 in the target protein's active site with lower binding affinity − 6.7 kcal/mol. The lowest binding score was displayed by compound **1** (–4.8 kcal/mol), while the best result was achieved using compounds **3** and **4** (–10.4 kcal/mol), with their affinity, found to be comparable to doxycycline (– 13.0 kcal/mol) and ciprofloxacin (– 8.4 kcal/mol) (Figure 2). The *in-silico* molecular docking analysis was also found to agree with *in-vitro* results. Hence, these compounds might have the potential to be promising antibacterial agents. The binding affinity, H-bond, and residual interaction of all the isolated compounds are summarized in Table 8.

Table 8. Molecular docking results of isolated compounds against *S. aureus* DNA Gyrase.

Ligands	Binding affinity (kcal/mol)	H-bond	Residual interactions	
			Hydrophobic and Electrostatic	Van der Waals
1	−4.8	Arg-458: HH11, Arg-458: CD, DC-12, DG-9	Hydrophobic-Pi-Sigma-DG-9; Hydrophobic-Pi-Sigma-DT-8; Hydrophobic-Alkyl-Arg-458; Hydrophobic-Pi-Alkyl-DG-9(Dist. 5.11974 Å); Hydrophobic-Pi-Alkyl-DG-9(Dist. 4.51935 Å) and Hydrophobic-Pi-Alkyl-DA-13	Asp-437, DT-10
2	−8.2	Glu-1088	Hydrophobic-Alkyl-Ala-1120(Dist. 4.63949 Å); Hydrophobic-Alkyl-Ala-1120(Dist. 4.22537 Å); Hydrophobic-Pi-Alkyl-DG-9(Dist. 5.37583 Å)	Arg-1122, Asp-1083, Met-1121, Ala-1119, Ser-1084,
3	−10.4	Asp-437, Gly-459	Hydrophobic-Pi-Sigma-Arg-DT-8;Hydrophobic-Pi-Sigma-DG-9(Dist. 3.44611 Å); Hydrophobic-Pi-Pi Stacked-DA-13; Hydrophobic-Pi-Alkyl-DT-8	Leu-457, Arg-458, Phe-1123, Arg-1122, Gly-436
4	−10.4	Gly-459, DG-9, DA-13, UNK0:H	Hydrophobic-Pi-Pi Stacked-DT-8(Dist. 3.96173 Å); Hydrophobic-Pi-Pi Stacked-DT-8(Dist. 5.03189 Å); Hydrophobic-Alkyl-Arg-458	DC-12
5	−6.7	Arg-76, Gly-77, Thr-165	Asn-46, Glu-50, Ile-78, Val-167	Asp-73, Ala-47, Gly-75, Val
6	−9.9	Gly-77, Glu-50	Asn-46, Arg-76, Ile-78, Pro-79	Asp-73, Ala-47, Thr-165
Doxycycline	−13.0	Ser-438, Ser-1084, Arg-1122	Electrostatic-Attractive Charge-Asp-1083; Hydrophobic-Pi-Alkyl-Ala-1120(Dist. 4.02007 Å); Hydrophobic-Pi-Alkyl-Ala-1120(Dist. 4.00436 Å)	Arg-1122, Met-1121, Phe-1123, Asp-437, DG-9
Ciprofloxacin	−8.4	Arg-1122, Asp-1083, Ala-1118, DG-9	Hydrophobic-Pi-Pi-T-Shaped-DG-9;Hydrophobic-Pi-Alkyl-DG-9;Hydrophobic-Pi-Alkyl-Ala-1120	DA-11, DT-10, Gly-1082, Phe-1123, Ser-438, Asp-437

The 2D and 3D binding interactions of compound **4** against *S. aureus* DNA Gyrase are depicted in Figure 2. 3D Ribbon and line models show the binding pocket structure of *S. aureus* DNA Gyrase with compound **4**. Hydrogen bonds between compounds and amino acids are shown as green dash lines, hydrophobic interactions are shown as pink/purple lines.

Figure 2. The 2D and 3D binding interactions of compound **4** against *S. aureus* DNA Gyrase.



3.6. Binding mode of isolated compounds docked against human topoisomerase IIβ

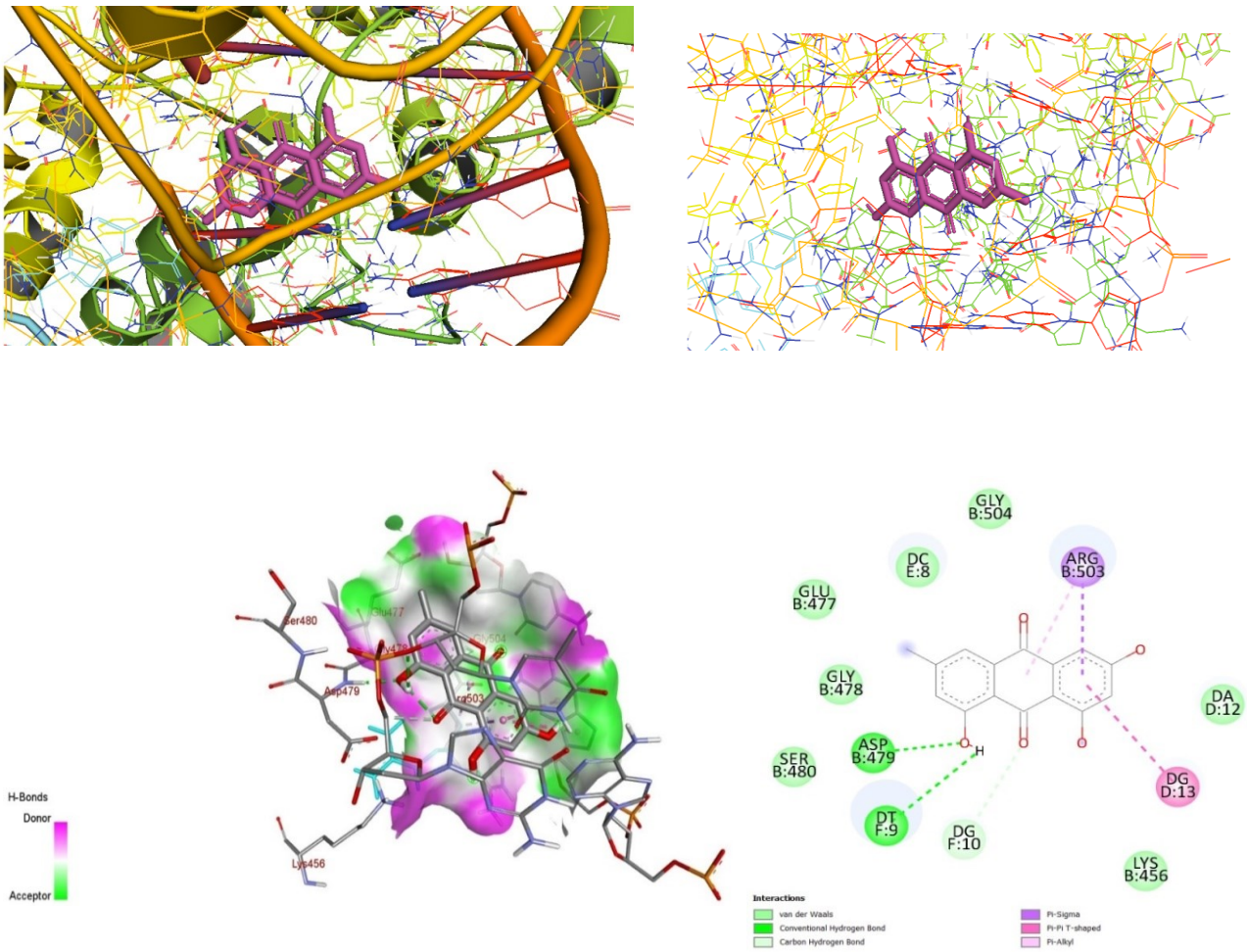
In this study, the molecular docking analysis of the isolated compounds was carried out to investigate their binding interaction within the binding sites of human topoisomerase IIβ and the results were compared with standard anti-cancer agents vosaroxin and abiraterone. The isolated compounds were found to have minimum binding energy ranging from – 5.6 to – 10.4 kcal/mol (Table 9). Compared with vosaroxin (– 10.2 kcal/mol) and abiraterone (– 11.8 kcal/mol), the isolated compounds have shown comparable binding affinity and residual and DNA interaction profiles with various amino acid residues. The *in-silico* interaction results have shown that the isolated compounds have a comparable binding affinity with abiraterone; among them, compounds **3** (– 9.8 kcal/mol) and **4** (– 10.4 kcal/mol) revealed good binding affinity (Figure 3). Compound **1** has shown lower docking affinity (–5.6 kcal/mol) but good matching amino acid residues interactions compared to vosaroxin and abiraterone. Based on the molecular docking analysis results, the isolated compounds have shown comparable residual interactions and docking scores with vosaroxin and abiraterone. Hence, compounds **3** and **4** might prove to be good anti-cancer agents. The binding affinity, H-bond, and residual interaction of all the isolated compounds are summarized in Table 9. However, *in-vitro* analysis has not been performed for anti-cancer activity, but promising *in-silico* results reflect that further analysis might be fruitful.

Table 9. Molecular docking results of isolated compounds against human topoisomerase IIβ.

Compounds	Binding Affinity (kcal/mol)	H-bond	Residual interactions	
			Hydrophobic	Van der Waals
1	− 5.6	Lys-456: HZ1, Lys-456: HZ3, DT-9	Hydrophobic-Alkyl-Arg-503 (Dist. 4.12106 Å); Hydrophobic-Alkyl-Arg-503 (Dist. 4.91145 Å); Hydrophobic-Pi-Alkyl-DC-8	Asp-479, Gly-478, Gly-504, DA-12
2	− 9.1	Asn-786, DG-5	Hydrophobic-Alkyl-Met-782(Dist. 4.0209 Å); Hydrophobic-Alkyl-Met-782(Dist. 4.73849 Å); Hydrophobic-Alkyl-Val-785(Dist. 4.37004 Å); Hydrophobic-Alkyl-Val-785(Dist. 5.11179 Å); Hydrophobic-Alkyl-Ala-816(Dist. 5.08237 Å); Hydrophobic-Alkyl-Ala-816(Dist. 4.35645 Å); Hydrophobic-Alkyl-Pro-819	Ser-818, Arg-820, Gln-778, Lys-814, Gly-813, Gln-789, Gly-812, DG-10, DC-11, DT-9, DA-12
3	− 9.8	Asp-479: HN, Asp-479: OD1, Arg-503, DT-9, DG-10, UNK0:H	Hydrophobic-Pi-Sigma-Arg-503(Dist. 3.835 Å); Hydrophobic-Pi-Sigma-Arg-503(Dist. 3.88167 Å); Hydrophobic-Pi-Pi T-Shaped-DG-13; Hydrophobic-Pi-Alkyl-Arg-503	Ser-480, Gly-478, Gly-504, Glu-477, DA-12, DC-8
4	− 10.4	Asp-479, DT-9, DG-10, UNK0:H	; Hydrophobic-Pi-Sigma-Arg-503; Hydrophobic-Pi-Pi T-Shaped-DG-13(Dist. 5.81293 Å); Hydrophobic-Pi-Pi T-Shaped-DG-13(Dist. 5.18989 Å); Hydrophobic-Pi-Alkyl-Arg-503	Gly-504, Glu-477, Gly-478, Ser-480, Lys-456, DC-8, DA-12,
Abiraterone	-11.8	DG-10	Hydrophobic-Alkyl-Arg-503(Dist. 4.50057 Å); Hydrophobic-Alkyl-Arg-503(Dist. 4.19301 Å); Hydrophobic-Pi-Alkyl-DA-12; Hydrophobic-Pi-Alkyl-DC-8	Gly-504, Glu-477, Gly-478, Lys-456, Asp-479, DT-9
Vosaroxin	− 10.2	Gln-778, DC-8, DG-10, DG-13	Pi-Sulfur-DT-9; Hydrophobib-Pi-Pi-Stacked-DT-9 (Dist. 5.68139 Å); Hydrophobib-Pi-Pi-Stacked-DT-9 (Dist. 4.27157 Å); Hydrophobic-Pi-Alkyl-Arg-503	Gly-776, DA-12, Lys-456

The 2D and 3D binding interactions of compound **4** against human topoisomerase IIβ (PDB ID 3QX3) are presented in Figure 3. 3D Ribbon and line models show the binding pocket structure of human topoisomerase IIβ with compound **4**. Hydrogen bonds between compounds and amino acids are shown as green dash lines, hydrophobic interactions are shown as pink lines.

Figure 3. The 2D and 3D binding interactions of compound **4** against human topoisomerase IIβ (PDB ID: 3QX3).



3.7. *In-silico* Pharmacokinetics (Drug-likeness) and Toxicity analysis

Drug-likeness is a prediction that determines whether a particular pharmacological agent has properties consistent with being an orally active drug. Lipinski's rule described that potential drug-like molecules should have the following properties: (i) less than five hydrogen-bond donors (HBDs), (ii) less than ten hydrogen-bond acceptors (HBAs), (iii) a molecular mass less than 500 Da, and (iv) log P not more than five and (v) total polar surface area (TPSA) should not be > 140 Å (Lipinski, Lombardo, Dominy, & Feeney, 2001). The results obtained in the present work revealed that all compounds satisfy Lipinski's rule of five (Table 10) (Ah & Yi, 2019). Hence, all the isolated compounds might be candidates for anti-cancer, antioxidant, and antimicrobial studies. Acute toxicity predictions result such as LD_{50} values, and toxicity class classification [1 (toxic) to 6 (non-toxic)] reveal that none of the ligands has shown acute toxicity and were found to be similar to standard drugs. The isolated compounds **1** and **2** have shown toxicity class classification 4 (harmful if swallowed), while **3** and **4** showed even better toxicity class 5. The toxicological prediction gives results of endpoints such as Hepatotoxicity, Carcinogenicity, mutagenicity, immunogenicity, and cytotoxicity. All the isolated compounds were predicted to be non-hepatotoxic, non-carcinogenic, non-irritant, and non-cytotoxic.

However, compounds **2** and **3** have shown immunotoxicity, while **3** and **4** have mutagenicity. Hence, based on ADMET prediction analysis, none of the compounds have shown acute toxicity, so that they might be proven as good drug candidates.

Table 10. Drug-likeness predictions of compounds, computed by SwissADME.

Ligands	Formula	Mol. Wt. (g/mol)	NRB	NHA	NHD	TPSA ($A^{°2}$)	Log *P* (iLOGP)	Log *S* (ESOL)	Lipinski's rule of five
1	$C_{12}H_{24}O_2$	200.32	10	2	1	37.30	2.70	-3.07	0
2	$C_{29}H_{50}O$	414.71	5	1	1	20.23	5.01	-7.99	1
3	$C_{15}H_{10}O_4$	254.24	0	4	2	74.60	2.22	-4.11	0
4	$C_{15}H_{10}O_5$	270.24	0	5	3	94.83	1.80	-3.67	0
5	$C_{13}H_{10}O_3$	214.22	0	3	2	49.69	2	-3.45	0
6	$C_{14}H_{10}O_5$	258.23	1	5	2	79.9	2.29	-3.37	0
Abirater-one	$C_{24}H_{31}NO$	349.51	1	2	1	33.12	3.42	-5.03	1
Doxycy-cline	$C_{22}H_{24}N_2O_8$	444.43	2	9	6	181.62	1.11	-2.59	1

NHD: number of hydrogen donors; NHA: number of hydrogen acceptors; NRB: number of rotatable bonds; TPSA: total polar surface area.

3.8. DFT Study

The structures of the isolated compounds, along with force on the nucleus, were optimized to find out the minimum global energy by the DFT. The energy difference between the Highest Occupied Molecular Orbital (HOMO) and the Lowest Unoccupied Molecular Orbital (LUMO) is an excellent indicator of electronic transition absorption in molecular systems. These provide insight into the reactivity of molecules. Owing to the HOMO–LUMO orbital interaction, LP-LP, and LP-bond pair type interactions were predominant in the investigated compounds. The calculated HOMO, LUMO energies, the energy gap, and dipole moment are shown in Table 11. The molecular orbital analysis for the investigated compounds based on their optimized geometry indicates that the frontier molecular orbitals are mainly composed of p-type atomic orbitals. An electronic system with a more significant HOMO-LUMO gap is less reactive than one with a smaller gap. Moreover, the HOMO–LUMO energy gap clearly explains the eventual charge transfer within the molecule. The power of an electronegative atom in a compound to attract an electron towards it was introduced by Pauling. The parameters such as hardness (η), ionization potential (I), electronegativity (χ), chemical potential (μ), electron affinity (A), global softness (σ), and global electrophilicity (ω) are calculated.

The ionization energy (IE) can be expressed through HOMO orbital energies, and electron affinity (EA) can be expressed through LUMO orbital energies. The hardness (η) corresponds to the HOMO and LUMO orbital energies gap, and the hardness has been associated with the stability of the chemical system. All the calculated values of quantum chemical parameters of the investigated molecules using the B3LYP method with 6-31G (d,p) basis-set are summarized in Table 11. From the results in Table 11, it is clear that for the molecules investigated, **4** has the minimum energy gap of 3.36138 eV, and **1** has the maximum energy gap of 7.58948 eV. These facts further indicate that compound **4** would be highly reactive among all the isolated compounds. All calculated values indicate the extensive charge delocalization in the investigated molecules, and the positive charges are localized over the hydrogen atoms.

The electron density is key to the bonding and geometry because the forces holding the nuclei together in a molecule are the attractive forces between the electrons and the nuclei. These attractive forces are opposed by the repulsion between the electrons and the repulsion between the nuclei. In the equilibrium geometry of a molecule, these electrostatic forces just balance. The fundamentally important Hellman–Feynman theorem (Popelier, 2000) states that

the force on a nucleus in a molecule is the sum of the coulombic forces exerted by the other nuclei and by the electron density distribution ρ. This distribution means that the energy of interaction of the electrons with the nuclei can be found by considering the classical electrostatic forces between the nuclei and the electronic charge cloud. The atoms are held together by the electrostatic force exerted by the electronic charge on the nuclei. Nevertheless, quantum mechanics, particularly the Pauli principle, determines the distribution of electronic charges. All these compounds have shown balanced charge distribution, which makes them adhesive to various biological enzymes.

Table 11. The various Quantum chemical parameters of isolated compounds.

S. No	Optimized energy (Hartree)	E_{HOMO} (eV)	E_{LUMO} (eV)	Energy Gap ΔE (eV)	Electro-negativity χ (eV)	Pauling Hardness η (eV)	Global Softness σ (eV^{-1})	Global Electrophilicity ω (eV)	Dipole Moment (Debye)
1	-622.2564	-7.2913	0.2982	7.5895	3.4966	3.7947	0.26352	1.6109	1.8706
2	-1210.479	-6.3799	0.7187	7.0986	2.8306	3.5493	0.28175	1.1287	1.3978
3	-878.5809	-6.4028	-3.0275	3.3753	4.7151	1.6876	0.59254	6.5869	1.0981
4	-953.8046	-6.3405	-2.9792	3.3614	4.6598	1.6807	0.59499	6.4599	2.4802

4. CONCLUSION

In this work, seven compounds such as lauric acid (1), *β*-stigmasterol (2), chrysophanol (3), emodin (4), 9H-Xanthene-3,5-diol (5), 1,7-dihydroxy-4-methoxyxanthone (6) and oleic acid (7) were isolated. Compounds **3** and **4** are new to the genus Laggera, while compounds **5** and **6** are new to the species *S. longipedunculata*. GC-MS analysis of the essential oil of the roots of *L. tomentosa* gave 2,5-dimethoxy-*p*-cymene as the principal constituent (59.39%), suggesting the use of the root of *L. tomentosa* as antibacterial agents. The antioxidant activity of the EtOAc extract, emodin (4), and 1,7-dihydroxy-4-methoxyxanthone (6) was found to be close to ascorbic acid, indicating the strong ability of the extract of the plant as radical scavengers and inhibitors of lipid peroxidation. The extracts of *L. tomentosa* and *S. longipedunculata* showed better antibacterial activity in a dose-dependent manner against *S. aureus*, with activity traced to compounds **4** and **6**, respectively. These compounds also displayed comparable binding affinities to ciprofloxacin and doxycycline against *S. aureus* DNA Gyrase, proposing that compounds **4** and **6** might be used as lead compounds for further development as antibacterial agents. Compound **4** also displayed a comparable affinity with vosaroxin and abiraterone against human topoisomerase IIβ. Hence, emodin (4) might be considered a lead compound for further development as an anti-cancer agent. Furthermore, the presence of emodin and chrysophanol and their immense biological activities demonstrate the positive attributes of the root of *L. tomentosa*.

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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

Yadessa Melaku, Tokuma Getahun, Markos Addisu and **Hailemichael Tesso** were responsible for the isolation, antibacterial, and antioxidant activities of the isolated compounds from *L. tomentosa* and *S. longipedunculata.* The *in-silico* molecular docking analysis and DFT

study were performed by **Rajalakshmanan Eswaramoorthy** and **Ankita Garg**. The write-up of the manuscript was done by all authors.

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Research Article

Phytochemical investigation of H*elianthemum lippii* l. aerial Dum.Cours part and evaluation for its antioxidant activities

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Abstract: Objective: The aim of this study is to determine the phytochemical contents and the antioxidant activity of Helianthemum lippii (L.) Dum.Cours. crude extract.
Methods: For preliminary phytochemical analysis, standard procedures were applied, while identification and quantification of individual phenolic compounds were performed by HPLC analysis. The Folin–Ciocalteu method was used to evaluate the total phenolic acid content of the plant extracts, The total flavonoid content was determined using the aluminum chloride colorimetric assay. The FTIR spectroscopy method was used to examine the chemical makeup of the organic extracts. The antioxidant activities were assessed using the 1,1-diphenyl-2-picrylhydrazyl and reducing power assays.
Results: Chemical analysis revealed the presence of numerous secondary metabolites, such as polyphenols, flavonoids, tannins, saponins, anthocyanins, cardiac glycosides, leuco anthocyanins steroids, terpenoids, alkaloids, and mucilage. For the HPLC analysis, we obtained 65 peaks and we identified 6 major elements of bioactive compounds. The total concentration of polyphenols and flavonoids was varied respectively 183.12±2.84 mg gallic acid eq/g dry wt and 72.00±1.03 mg quercetin eq/g dry wt /mg. The general concentration of condensed tannin and hydrolyzable tannin compounds were expressed in terms of catechin equivalent (5.88±1.58 mg Ca eq/g dry extract) and gallic acid (2.818±0.138 mgTA eq/g dry wt) respectively. FTIR spectroscopy investigation indicated several characteristic peak values in the extract with diverse functional groups such as amide, alcohol, and phenol groups. Concerning the antioxidant activity, we found that this extract has high inhibitory percentages equivalent to IC50 3.085±0.001 for DPPH and 1.724±0.021 for reduction power (µg/mL).
Conclusion: Our study proved that the aqueous extract of the H lippii is very rich in secondary metabolites; in addition, it has a tremendous anti-oxidant capacity, which leads us forward to introduce it for medical use.

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1. INTRODUCTION

Medicinal plants and their therapeutic properties are widely used around the world for a variety of disorders (Ahmad *et al*., 2014; Mohammed, Kına, *et al*., 2021; Sevindik *et al*., 2017). Herbal medicine is one of the most important branches of traditional medicine all over the world; it is made up of phytochemicals that help people to restore and improve physiological balance. The

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knowledge of these medicinal properties has been passed down through generations (Chaouche *et al*., 2020). Secondary metabolites form a series of active substances for the treatment of some human diseases (Ali-Rachedi *et al*., 2018; Uysal *et al*., 2021).

Phytochemicals with anticarcinogenic and antibacterial capabilities are currently gaining popularity 1-3 in a variety of fields, including agriculture, food, and preventive and therapeutic medicine. Plants biosynthesize a diverse spectrum of phytochemical compounds, which are primarily employed in the discovery and development of novel medications (chemotherapeutics). The food sector, on the other hand, is getting more interested in plants that can be eaten and that contain health-promoting chemicals, notably those high in phenols, which have antioxidant characteristics (Mubashar Sabir *et al*., 2015). Polyphenols compounds possess a tremendous capacity for natural antioxidants, which have the potential to scavenge free radicals and protect cells from oxidative damage (Rotta *et al*., 2017). As a result, Polyphenols or plant extracts high in phenols could be used as active biocapacity elements diets to aid in the prevention of oxidative stress-related disorders.

As a result, researchers exploring food additives, nutraceuticals, and diet supplements are looking for new plant sources of antioxidants inclusive edible and non-edible plants are high in phenols (Ćetković *et al*., 2004; Rotta *et al*., 2017). Plant phenols play an important role act as free radical scavengers, reducing Fe+3/Fe+2, inhibiting peroxidation, and having an anti-inflammatory effect through reducing cytokine expression has been linked to a lower risk of cardiovascular and chronic disease, as well as certain cancer. All of this has been proven in previous studies (Farahpour, 2014; Girola *et al*., 2015).

This study was designed specifically for the purpose of conducting a phytochemical screening of *Helianthemum lippii*, and to determine the total phenolic, flavonoid, and total tannins content, and FT-IR spectrum is used to validate the presence of organic functional groups. In order to verify the antioxidant capacity of the extract, we used methods DPPH and reduction power.

2. MATERIAL and METHODS

2.1. Preparation of Plant Material

The aerial parts of the *H. lippii* were collected during the flowering period in March 2020 in Southeastern Algeria (exactly in the region of Elhamadin- province of El -Oued), the plant material was identified by Professor Atef CHOUIKH (Faculty of Natural Science and Life, El Oued University). To eliminate dust and other foreign particles, the aerial section was cleaned under running tap water. Then, it was dried, ground, and stored for future use.

2.2. Chemicals

This investigation employed only analytical-grade compounds, Folin-Ciocalteu reagent, Gallic acid, Quercetin, Tannic acid and Ascorbic acid (Vitamin C), Sodium carbonat, Aluminium chlorid, ferric chloride, Potassium ferricynide, trichloroacetic acid, vanillin, hydrochloric acid, 2,2_-diphenyl-1-picryl-hydrazyl (DPPH), and all other reagents of phytochimical analytical Chemicals were received from Sigma Aldrich Co (St. Louis, MO, USA).

2.3. Preparation of Aqueous Extract

H. lippii aerial parts powder was steeped for roughly 10 g in 100 mL distilled water and left at room temperature for 24 hours in the dark. After that, it was filtered with filter paper. After extraction, the sample was dried extensively at 40°C. The extract was weighed and refrigerated at 4°C for further analysis in the future (Murugan & Parimelazhagan, 2014).

2.4. Phytochemical Screening

The extract was tested to detect the different compounds such as; phenols, cardiac glycosides, tannins (catechetical tannins. and gallic tannins), alkaloids, steroids, saponins, flavonoids, triterpenoids, leuco anthocyanins, anthocyanins, and of mucilages employing usual standard procedures for phytochemical analysis (screening)(Matos, 1997). The presence of phytochemicals is indicated by a (+) while the absence of phytochemicals is indicated by a (-).

2.5. Analyze Qualitative by HPLC

Using scanning equipment and high-performance liquid chromatography, the active components were discovered (HPLC). For the investigation of phenolic chemicals in crude extract, we utilized HPLC with UV-Vis type Shimadzu LC20 AL equipped with the universal injector (Hamilton 25l), an analytical column was a Shim-pack VP-ODSC18 (4,6mm, 250mm, 5m), and UV-VIS detector SPD 20A type (Shimadzu). The reverse-phase chromatography studies were conducted using non-polar aliphatic residues, and the mobile phase was comprised of gradient elution of a combination of acetonitrile and acetic acid (0.1%). The injection volume was 0, 45µL and the flow rate was 1mL/min. The monitoring wavelength was 268nm, and the sample and standard injection volume were 20µL. The retention duration and UV absorbance of various compounds were compared to those of the standards to identify them.

2.6. Quantification of Phytochemical Compounds

2.6.1. Estimation of total phenolics

Total phenolic content was calculated using the Folin–Ciocalteu method (Li *et al*., 2007). (0.2 mL) of the sample of the aqueous extract of *Helianthemum lippii* and 1mL of Folin–Ciocalteu reagent were added diluted of 1:10 and added 800 µL of saturated sodium carbonate (7,5%) after 4 min. The absorbance was measured at 765 nm after 2 hours of incubation at room temperature. The tests were repeated three times to confirm that the results were consistent. The total phenolic content of the extract was measured in milligrams of gallic acid equivalent per gram of extract.

2.6.2. Estimation of total flavonoids

1 mL of the $AlCl_3$ solution is mixed with 1 mL of the sample, and on other hand with 1 mL of the standard. The absorbance was measured at max = 430 nanometers after 10 minutes against the prepared reagent blank (Ahn *et al*., 2007). The results were expressed in milligrams of Quercetine per gram of extract.

2.6.3. Estimation of total hydrolysable tannins

The Folin-Ciocalteu colorimetric method was used to calculate the total hydrolysable tannin concentration. A 10 mL test tube containing 8.4 mL distilled water, 0.5 mL Folin-Ciocalteu reagent, and 0.1 mL 7 percent Na_2CO_3 solution was filled with an aliquot of 1 mL tannic acid in distilled water of each concentration. Absorbance was measured at 700 nm against a blank after incubation for 30 minutes. All of the tests were repeated three times. Tannic acid equivalents (TAE) per gram of dry extract (mg/g) were used to assess the total tannin content of the extract (Poudel & Rajbhandari, 2020).

2.6.4. Estimation of condensed tannins

The extract's tannin concentration was determined using spectrophotometry (Broadhurst & Jones, 1978).The calibration curve was created using catechin. 0.5 mL sample pipetted into an aluminum foil tube, mixed with 3.0 mL vanillin reagent (freshly made 4 percent w/v vanillin in methanol), and 1.5 mL concentrated hydrochloric acid, and thoroughly mixed. The absorbance was measured against water at 500 nm after 15 minutes of reaction at 20-2°C.

2.7. Characterization of Heluintium liippii L. Aqueous Extract by Fourier Transforms Infrared (FTIR)

Fourier transform infrared spectroscopy analysis: The FT-IR spectrum was produced using a spectrophotometer (Shimadzu-00463 model) with a resolution of 4 cm^{-1} and 64 coadded scans in the spectral region of 4000-400 cm^{-1}. The surface chemistry and organic functional group are confirmed by the FT-IR spectrum.

2.8. Antioxidant Activity

2.8.1. DPPH free-radical scavenging activity

2.4 mg of DPPH• is dissolved in 100 mL of methanol to make the 1,1-diphenyl-2-picrylhydrazyl solution. 1mL of each phenolic extract (or ascorbic acid as a control) is added to 1 mL of the DPPH• solution previously produced. The reaction mixture is quickly agitated and then maintained at room temperature for 30 minutes in the dark to complete the reaction. The reaction medium's absorbance is measured at 517 nm (Mansouri *et al*., 2005).

2.8.2. Reducing power assay

The reducing power of the extract was determined using Oyaizu's methods (Oraiza, 1986). In distilled water, the extract was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1 percent potassium ferricyanide water solution (2.5 mL, K3 [Fe (CN) 6]) at different concentrations (mg/mL). The mixture was incubated at 50°C for 20 minutes; the mixture was centrifuged for 10 minutes at 3000 rpm after Aliquots of trichloracetic acid (2.5mL, 10% aqueous solution) were added. A freshly produced FeCl3 (0.5mL, 0.1%) solution was combined with the supernatant (2.5mL) and purified water (2.5mL) At 700 nm, the absorbance was measured. A positive control was employed, which was ascorbic acid.

2.9. Statistical Analysis

All of the experiments were carried out in threes. The data were analyzed in Microsoft Excel and are presented as mean ± standard deviation (n = 3). Graphpad Prism 7 for Windows was used to calculate the IC_{50} and EC_{50} values.

3. RESULTS

3.1. Phytochemical Screening

The results of the phytochemical tests reveal the richness of the extract by various active constituents. The main active constituents of *H.lippii* are polyphenols, flavonoids, tannins, saponines, anthocyanins, cardiac glycosides, leuco anthocyanins steroids, terpenoids, alkaloids, and mucilage (Table 1).

Table 1. Phytochemical screening of *H.lippii* aqueous extract.

Phytochemical compounds		*Helianthemum lippii* (aqueous extract)
Polyphenols		(+)
Alkaloids	Mayer	(-)
	Wagner	(+)
Tannins	Catechin	(+)
	Gallic	(+)
Flavonoids		(+)
Saponines		(+)
Anthocyanins		(+)
leuco anthocyanins		(+)
Cardiac glycosides		(+)
Steroids and terpenoids		(+)
Mucilage		(+)

(−) Absence of phytochemicals compounds.
(+) Presence of phytochemicals compounds.

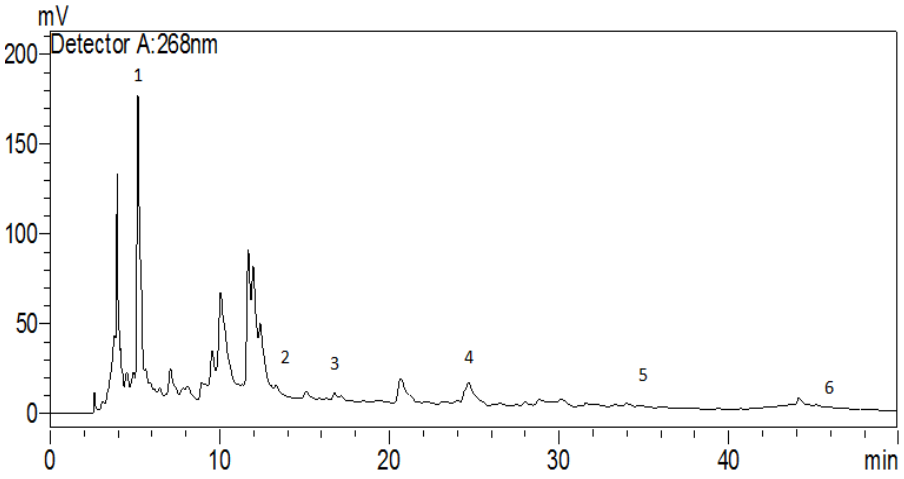
3.2. Analysis Qualitative by HPLC

The results of the separation of the aqueous extract by the use of HPLC are illustrated in the Helianthemum lippii chromatogram (Figure 1). Where we have identified six phenolic compounds out of 65 peaks. The analysis revealed that gallic acid (9495.115305 µg/g) was the most abundant element with a high amount of chlorogenic acid (7107.242096 µg/g), a moderate quantity of quercetin (1118. 647803 µg/g), a little amount of Naringin (738.1908251 µg/g), p-coumaric acid (663.776139 µg/g), and caffiec acid (444.8195465 µg/g) were detected in *H.lippii*. The results are represented in Table 2.

Table 2. Retention time and the concentration of phenolic compounds identified in *Helianthemum lippii* aqueous extract.

Phenolic compound	Retention Time (min)	Concentrction (µg/g extract)
Gallic Acid	5.29	9495.115305
Chlorogenic Acid	13.392	7107.242096
Caffiec Acid	16.277	444.8195465
p-Coumaric Acid	23.817	663.776139
Naringin	34.788	738.1908251
Quercetin	45.047	1118.647803

Figure 1. HPLC chromatogram's of the extract of *Helianthemum lippii:* 1: Gallic Acid; 2 Chlorogenic Acid; 3: Caffiec Acid; 4: p-Coumaric Acid; 5: Naringin; 6: Quercetin.



3.3. Quantification of Phytochemical Compounds

The total phenol and total flavonoid content were determined in comparison with a standard which is equivalent (mg GA eq/g dry extract) gallic acid and quercetin equivalent (mg Q eq/g dry extract) respectively, using the following equations based on the calibration curve: y = 0.0104x+0.0819, R^2= 0.9925 for total phenol and y =0.0096x+0.0521, R^2= 0.994 for total flavonoid.

Whereas, total condensed tannin and total hydrolyzable tannin compounds were calculated by Catechin equivalent (mg Ca eq/g dry extract) and gallic acid (mgTA eq/gdry wt) respectively, using the following equations based calibration curve: y = 0.0005x-0.0052, R^2 = 0.9885 for condensed tannin and y = 0.0482x – 0.0522, R^2 = 0.9377 for total hydrolysable tannin compounds. The results were represented in Table 3.

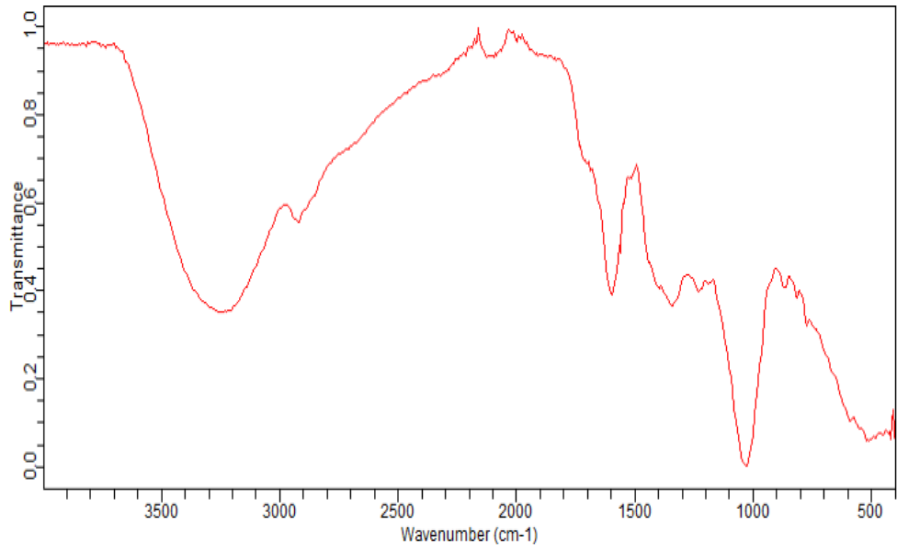
Table 3. Quantitative analysis of total phenolic, flavonoid and total tannins compounds of *Helianthemum lippii* aqueous extract.

Extract	Total phenolic (mgGAE/g extract)	Total flavonoid (mgQE/mg extract)	Condensed tannin (mg Ca eq/g dry wt)	Total hydrolysable tannin (mgTA eq/gdry wt)
H.lippii	183.12±2.84	72.00±1.03	5.88±1.58	2.818±0.138

3.4. Characterization of *Helianthemum lippii* Aqueous Extract by Fourier Transforms Infrared (FTIR)

Based on the infrared radiation region's highest value, the FTIR spectrum was utilized to determine the active component's functional group. The most prominent FTIR peak values and functional groups are shown in the results (Figure 2). The presence of a hydroxyl group at 3201.07 cm^{-1} was discovered in the profile of FTIR spectra of aqueous extract of *H. lippii.* The stretching vibration of C=C is responsible for the steep peak at 1599.49 cm^{-1}. Finally, our plant's C-O function produces a sharp band at 1078 cm^{-1}.

Figure 2. Infrared spectrum of *Helianthemum lippii* aqueous extract.



3.5. Antioxidant Activity

We measured the relative antioxidant ability of our sample by using DPPH and Reducing power assays. The extract of *H.lippii* has a very high antioxidant capacity through the values of IC50 and EC50 values for DPPH and reduction power activities which were respectively 3.085±0.001µg/mL and 1.724±0.021µg/mL Table 4.

Table 4. Antioxidant activities of aerial part of *Helianthemum lippii.*

Extracts/standards	DPPH· (IC_{50}: µg/mL)	Reduction Power (EC_{50}: µg/mL)
H.lippii	3.085±0.001	1.724±0.021
Ascorbic acid	1.219±0.005	0.225±0.032

4. DISCUSSION

Medicinal plants contain active ingredients, according to extensive research. This bioactivity is caused by a variety of antioxidant-rich phytochemicals found in medicinal plants. The phytochemical content and antioxidant activity of crude extract from *H. lippii* species are studied in this paper. An examination of the Phytochemical indicated the presence of phenols,

cardiac glycosides, tannins (catechetical tannins and gallic tannins), flavonoids, steroids, triterpenoids, alkaloids, saponins, anthocyanins, leuco anthocyanins, and mucilages. These substances are biologically active and have the potential to improve *H. lippii*'s antioxidant capabilities. This plant extract has antioxidant properties due to because of the existence of terpenoids and flavonoids.

Gallic acid was found to be the most abundant phenolic ingredient in the *H.lippii* extract, which was quantified by HPLC, followed by chlorogenic acid, caffiec acid, p-coumaric acid, naringin, and quercetin. These compounds were also demonstrated to have the ability to scavenge free radicals (Rotta *et al*., 2017).

The study has shown that the *H. lippii* contains a high quantity of phenolic and flavonoid compounds and a slight amount of tannins. The antioxidant activity of this plant is due to the presence of phenolic and flavonoid content imoprtant. Many investigations have discovered that phenolic compounds also have biological effects, as for example anti-inflammatory properties, in addition to antioxidant, antimicrobial,antiulcerogenic, and even cancer-fighting capabilities (Alarcón *et al*., 2008; Carro *et al*., 2016; Głód *et al*., 2015 ;Granato *et al*., 2013; Kına *et al*., 2021; Mohammed, *et al*., 2021; Pehlivan *et al*., 2021; Valdés, 2015).

There is a lot of interest these days in measuring the ability of plant or food extracts to function as antioxidants to see if they have any therapeutic qualities. One of the methods utilized to assess antioxidant activity was the DPPH assay, which is one among the most strong, simple, and reliable in vitro methods for sequestering free radicals. The total phenolic content of plant extracts is frequently linked to antioxidant activity (Ghani *et al*., 2019). They can react with hydroxyl, superoxide anion, and lipid peroxyl radicals, which are all active oxygen radicals to early detection and prevention of lipid peroxidation. The hydroxyl groups in phenolic acids are responsible for their high scavenging activity (Bruck de Souza *et al*., 2020; Cai *et al*., 2003; Miliauskas *et al*., 2004). Because a compound's reducing power is connected to its ability to move electrons, it can be used to predict its prospective antioxidant activity. This can be linked to the polyphenols in the extract's propensity to donate electrons (Aluko, 2017).

Fourier Transform Infrared Spectroscopy (FTIR) is a non-destructive characterization technique that uses infrared light to irradiate the sample, and the absorbed energy gives a specific spectrum depending on the chemical composition of the sample. The absorption signals for diverse wavenumber ranges in the FTIR spectrum produced in this investigation revealed varied typical peak values with various functional groups in the extract such as alcohol, amide, and amino acids compounds.

5. CONCLUSION

According to this study, the crude extract of H.lippii is extraordinarily rich in phytochemicals and antioxidants of high medicinal importance, verifying and validating its use in the treatment of a variety of human illnesses. Consequently, the extract’s chemical composition, mainly phenolic compounds, may have a synergistic impact. As a result, our findings pave the way for additional detailed examinations of the extract’s mechanisms of action at a higher level of cellular and organism complexity for its medicinal evaluation.

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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

Ibtissam Laib: Investigation, Visualization, Conceptualization Ideas; formulation or evolution of overarching research goals and aims, design of methodology, and Writing the manuscript. **Ali Boutlelis Djahra**: Supervision, Methodology, and Validation.

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Research Article

The Use of Organic Sun-Dried Fruits for Delivery of Phenolic Compounds

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Abstract: The aim of this study is to characterize and increase the total soluble (water soluble + alcohol soluble) phenolic (SPC_T) and flavonoid content (SFC_T) and total soluble free radical scavenging based antioxidant capacity (SAC_T) of major sun-dried fruits such as raisins, figs, prunes and apricots. Due to their high insoluble dietary fiber content, the bound antioxidant capacity formed 61 to 67% of the overall antioxidant capacity (water soluble + alcohol soluble + bound) of sun-dried fruits. The SPC_T, SFC_T and SAC_T of sun-dried fruits changed between 1675 and 3860 µg catechin/g (d.w.), 161 and 495 µg catechin/g (d.w.) and 13 and 28.5 µmol Trolox/kg (d.w.), respectively. The incorporation of green tea polyphenols into sun-dried raisins, figs and apricots by controlled rehydration conducted in green tea extracts increased their SPC_T, SFC_T and SAC_T 1.5 to 1.8 fold, 1.3 to 1.6 fold, and 1.5 to 2.6 fold, respectively. The method applied caused limited increases in SPC_T (1.1 fold) and SFC_T (1.2 fold) of prunes, but it increased SAC_T of these fruits 1.6 fold. This study showed the possibility of using sun-dried fruits not only as source of dietary fiber, but also for delivery of phenolic compounds. The methods used in this study for delivery of green tea phenolic compounds to selected organic sun-dried fruits could be an alternative method to increase intake of these invaluable antioxidant compounds and increase functionality of sun-dried fruits which are already accepted as good source of dietary fiber.

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1. INTRODUCTION

The dried fruits have attracted an increasing interest since they are invaluable sources of dietary fiber. There is an increasing consensus among scientists that a fiber-rich diet might have important metabolic effects to reduce cardiovascular diseases, diabetes and cancer (Kendall *et al.*, 2010). The pectin found in most dried fruits is a functional fiber that exists both in soluble and insoluble forms. The viscous fibers like soluble pectin are important since they increase the consistency of intestine contents, slow down digestion, and reduce the amount of glucose and cholesterol diffused from the intestinal lumen (Kendall *et al.*, 2010). On the other hand, the insoluble pectin prevents constipation by hydrating and increasing movement of waste through intestine. The organic sun-dried fruits are particularly attractive source of dietary fiber since

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they are free from pesticides, hormones and other chemical contaminants, and dried without using fossil energy.

In addition to dietary fiber, sun-dried fruits also contain polyphenols which are known with their health benefits and show preventive effects on major diseases. The phenolic compounds owe their health benefits mostly to their antioxidant activity that is mainly originated from their free radical scavenging capacity (Blažeković *et al.*, 2012). The frequent and long term intake of phenolic compounds by consumption of fresh fruits and vegetables helps improving pro-oxidant/antioxidant balance which plays important role in immune system to suppress oxidative stress and its damages on cells. Thus, it is accepted that the phenolic antioxidants provide protection against many major diseases including cardiovascular diseases, cancer, diabetes, neurodegenerative disorders, autoimmune disorders, and aging (Blažeković *et al.*, 2012). Dried fruits including raisins, prunes, figs and apricots are good sources of antioxidant phenolic compounds. A recent study demonstrated that raisins are good dietary source of flavonols and phenolic acids (Karadeniz *et al.*, 2000; Williamson & Carughi, 2010). The dried figs are good sources of phenolic compounds such as proanthocyanidins, flavonols, flavones and phenolic acids (Vallejo *et al.*, 2012) while dried prunes and apricots are rich mainly in phenolic acids (Chang *et al.*, 2016). However, it has been recently reported that the polyphenols in dietary fiber rich fruits might have low bioaccesibility and bioavailabilities (Palafox-Carlos *et al.*, 2011). The polar groups of carbohydrates that form majority of dietary fiber could bind phenolic hydroxyl groups with non-covalent interactions such as hydrogen bonds and van der Waals forces (Barros *et al.*, 2012; Palafox-Carlos *et al.*, 2011; Wu *et al.*, 2009). Thus, it is thought that the binding of the phenolic compounds by dietary fiber could prevent the adsorption of phenolic compounds in stomach and small intestine. The pectin-like polysaccharides that are normally accepted as invaluable dietary fibers reduce bioaccesibility and bioavailability not only for the phenolic compounds, but also for carotenoids by entrapping these antioxidants and reducing their diffusivity and contact with other essential components necessary for their absorption (Palafox-Carlos *et al.*, 2011; Pandey & Rizvi, 2009). The *in-vivo* data about the bioavilability of phenolics from sun-dried fruits are scarce, but recent *in-vitro* studies of Kamiloğlu *et al.* (2014) showed that the bioaccessible phenolic contents of sun-dried apricots, raisins and figs could be only between 8 % and 19 % of total phenolic compounds following gastric digestion. The binding of antioxidant phenolic compounds by food hydrocolloids including dietary fiber is also an important problem limiting bioaccesibility and bioavailability of phenolics in legumes and cereals. It was reported that 25 to 85% of total antioxidant activity might be formed by bound phytochemicals in legumes such as lentils, chickpeas, yellow and green beans, and soybeans (Han & Baik, 2008). Serpen *et al.* (2007) reported that 50% of the total antioxidant activity in cereal based food is formed by bound antioxidants. Thus, the enrichment of fiber rich food by phenolic compounds and increase of their soluble phenolic content and antioxidant activity could be a good strategy to improve their functional properties.

In this work the soluble and bound free radical scavenging based antioxidant capacity of sun-dried fruits such as raisins, figs, apricots and prunes were characterized, and green tea polyphenols were incorporated into sun-dried fruits by controlled rehydration conducted in solutions of green tea extracts. The green tea is one of the most abundant and popular sources of phenolic compounds. Thus, catechins ($C_6C_3C_6$), the major phenolic constituent in green teas, are among the most thoroughly characterized phenolic class in the literature for their molecular structure (Zaveri, 2006), bioavailability and *in-vivo* and *in-vitro* health benefits (Shimizu & Weinstein, 2005; Thielecke & Boschmann, 2009). Recently, dried fruits have been used for delivery of probiotics (Betoret *et al.*, 2003; Rêgo *et al.*, 2013). This study aimed at improving benefits from sun-dried fruits and using them for the first time for delivery of phenolic compounds.

2. MATERIAL and METHODS

2.1. Materials

The sun-dried organic fruits, figs (Cultivar Sarılop), plums (red colored plums), raisins (sultanas) and apricots (non-sulfited apricots), were purchased from Işık Tarım Ürünleri Sanayi ve Ticaret A.Ş in İzmir (Turkey). The organic green tea was provided by the Beta Tea (Turkey). ABTS (2,2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Aluminum chloride phosphate and Folin-Ciocalteu's phenol reagent were purchased from Fluka (Switzerland).

2.2. Preparation of Green Tea Extract

The green tea extract was prepared by mixing 50g of green tea with one hundred milliliters of distilled water at 85 °C for 20 minutes under continuous magnetic stirring. The green tea infusion obtained was then filtered from cheese cloth to remove the debris and clarified by centrifugation at 15000 x g for 15 minutes. Centrifuged extract was lyophilized and stored at -18 °C until it was used in phenolic enrichment studies.

2.3. The Use of Green Tea Extract in Phenolic Enrichment of Sun-Dried Fruits

The incorporation of green tea polyphenols into sun-dried fruits was conducted by 3h rehydration of fruits in 1% (w/w) solutions of green tea extract at room temperature (fruit/extract ratio (w/v): 1/7.5). Controls were rehydrated in distilled water at the same conditions. After rehydration, the free water at the surface of fruits was removed using a filter paper and the fruits were weighted to determine their green tea extract intake. The moisture contents of the fruits were determined by the standard vacuum oven method (AOAC 1996). The final moisture contents of raisins, figs, apricots and prunes rehydrated in green tea extract were 39%, 40%, 44% and 37%, respectively.

2.4. Extraction of Soluble Phenolic Compounds from Dried Fruits

The extraction of water soluble phenolic compounds was conducted by homogenization of 20 g rehydrated fruits with 150 mL distilled water using a blender equipped with a stainless still jar (Waring blender, USA) for 2 min. The obtained slurry was further homogenized with a homogenizer-disperser (IKA, DI 18, Basic, Brasil) at 18000 rpm for 2 min and it was centrifuged at + 4°C for 30 min at 15000 x g. The supernatant (S1) was separated and the collected pellet was once more homogenized with an additional 150 mL distilled water using the homogenizer-dispenser at 18000 rpm for 2 min. The homogenate was then centrifuged at + 4°C for 30 min at 15000 x g. The supernatant (S2) was combined with S1 and the pellet (P1) separated for extraction of alcohol soluble phenolic compounds. The combined extract containing S1 and S2 was named as water soluble extract.

The extraction of alcohol soluble phenolic compounds was conducted by mixing the 2 times water extracted pellet (P1) with 150 mL of ethanol and conducting homogenization with the homogenizer-disperser at 18000 rpm for 2 min. The homogenate was clarified by centrifugation at +4°C for 30 min at 15000 x g for 30 min and the supernatant (S3) containing the hydrophobic (lipophilic) phenolic compounds was named as alcohol soluble extract. The pellet (P3) collected from the centrifugation was separated and used in bound antioxidant activity determination.

2.5. Determination of Total Soluble Phenolic Content

The phenolic content of water and alcohol soluble extracts were determined using the Folin-Ciocalteu method according to Singleton and Rossi (1965). A 0.2 mL sample of appropriately diluted extract was mixed with 1 mL of 1/10 diluted Folin-Ciocalteu reagent. After 3 minutes incubation, 0.8 mL of a 7.5 % Na_2CO_3 solution was added to the mixture. The final mixture was further incubated for 2 hours and its absorbance at 765 nm was measured with a spectrophotometer (Shimadzu, UV-VIS Model 2450, Japan). Total phenolic contents of

samples were expressed as milligrams of catechin equivalents per kg dry weight (d.w.). All measurements were conducted five times. The total soluble phenolic content (SPC_T) was determined by finding the sum of phenolic contents of water soluble extract (SPC_W) and alcohol soluble extract (SPC_A) for each fruit.

2.6. Determination of Total Soluble Flavonoid Content

The flavonoid content of water and alcohol soluble extracts were determined using the method described by (Jia *et al.*, 1999). Before analysis 250 µl of extract was diluted with 1 mL of distilled water. Then, 75 µl of 5 % $NaNO_3$ was added onto the diluted sample. The reactants were mixed and incubated for 5 min. Then, 75 µl of 10% $AlCl_3$ was added onto the mixture and it was further incubated for 1 min. At the end of the incubation period, 0.5 mL of 1 M NaOH solution and 0.6 mL distilled water were added onto the final mixture and its absorbance was determined at 510 nm. The total flavonoids content was expressed as milligrams of catechin equivalents per kg d.w. All measurements were conducted five times. The total soluble flavonoid content (SFC_T) was determined by finding the sum of flavonoid contents of water soluble extract (SFC_W) and alcohol soluble extract (SFC_A) for each fruit.

2.7. Determination of Total Soluble Antioxidant Capacity

The free radical scavenging based soluble antioxidant capacities (SAC) of samples were determined using the classical ABTS method given by (Re *et al.*, 1999). The ABTS free radical solution was obtained by treating 7 mM ABTS solution with 2.45 mM potassium persulfate. The ABTS was diluted with 5 mM pH 7.4 phosphate buffer containing 150 mM NaCl (PBS) until its absorbance reached 0.70 spectrophotometric absorbance units at 734 nm. The reaction mixture was prepared by mixing 25, 50 and 75 µl of extract with 2 mL of ABTS solution. The absorbance of each reaction mixture was then monitored and recorded after 1, 3, 6, 9, 12 and 15 min. To calculate the AUC, the percent inhibition/concentration values for the extracts were plotted separately against test periods. All measurements were conducted three times and antioxidant activity was expressed as Trolox equivalents (µmol) per kg dry weight. The total soluble antioxidant capacity (SAC_T) was determined by finding the sum of antioxidant capacities of water soluble extracts (SAC_W) and alcohol soluble extracts (SAC_A).

2.8. Determination of Total Bound Antioxidant Capacity

The total bound antioxidant capacities (BAC_T) of samples were determined according to (Arda Serpen *et al.*, 2007) with some minor modifications. The BAC_T was determined using water and ethanol extracted pellet (P3). The pellet was prepared for measurements following 2 times additional washing with 2 x 40 mL ethanol. Each washing was conducted under continuous magnetic stirring at 500 rpm for 30 min and pellet was collected each time by centrifugation at 15000 x *g* for 10 min. The washed pellet was then lyophilized and used in BAC_T measurements. The tests were conducted by mixing 20 mg lyophilized pellet with 1.9 mL of ABTS solution and incubating this reaction mixture for 12 min at 30°C under continuous shaking at 150 rpm. The absorbance of the reaction mixture was measured at 734 nm after centrifugation of reaction mixture at 6000 x g for 2 min. All measurements were performed five times and antioxidant activity was expressed as Trolox equivalents (mmol) per kg d.w.

2.9. Determination of Overall Antioxidant Capacity

The overall antioxidant capacity (OAC) was determined by finding the sum of SAC_T and BAC_T.

2.10. Statistical Analysis

The statistical analyses were carried out by using ANOVA with analyzing data for the analysis of variance. Values were significantly different at $p<0.05$ as determined by Fisher's protected least significant difference.

3. RESULTS and DISCUSSION

3.1. Phenolic and Flavonoid Contents of Organic Sun-Dried Fruits

The SPC_T and SFC_T of organic sun-dried raisins, figs, prunes and apricots were given in Table 1. The SPC_T and SFC_T of sun dried fruits varied between 1675 and 3860 µg catechin/g (d.w.), and 161 and 495 µg catechin/g (d.w.), respectively. The prunes had the highest SPC_T that was 1.9 to 2.3 fold higher than those of the other fruits. The SPC_T values of figs and apricots were quite similar, and these fruits contained slightly higher (almost 1.2 fold) SPC_T than that of raisins. In all sun-dried fruits, the water soluble phenolic content (SPC_W) formed 92 to 96 % of SPC_T while the remaining minor fraction in the water extracted fruits was formed by alcohol soluble phenolic compounds (SPC_A). The results for SPC_W showed a high parallelism with those of SPC_T. The prunes showed 2.0 to 2.4 fold higher SPC_W than the other fruits. The figs and apricots contained similar SPC_W values while raisins showed almost 1.2 fold lower SPC_W than these fuits. On the other hand, it should be reported that there was no statistically significant difference between SPC_A values of prunes and figs, but these fruits showed 1.3 to 1.4 fold higher SPC_A than raisins and apricots which also showed similar SPC_A values.

Table 1. Total soluble phenolic and flavonoid contents of different organic sun-dried fruits.

Product	Phenolic Content (µg catechin/g d.w.)			Flavonoid Content (µg catechin/g d.w.)		
	SPC_W	SPC_A	SPC_T	SFC_W	SFC_A	SFC_T
Raisin	1555 ±30.5^{c}	120 ±7.0^{b}	1675	120±3.74^{b}	107 ±4.7^{b}	227
Fig	1790 ±36.0^{b}	150 ±31.0^{ab}	1940	315±30.0^{a}	180 ±22.9^{a}	495
Prune	3690 ±50.0^{a}	170 ±4.5^{a}	3860	328 ±11.9^{a}	64 ±5.3^{c}	392
Apricot	1895 ±38.5^{b}	135 ±4.0^{b}	2030	72 ±2.6^{c}	89 ±1.5^{b}	161

$^{a-c}$ Values followed by different letters are significantly different at $p<0.05$.

The figs showed the highest SFC_T that was almost 1.3, 2.2 and 3.1 fold higher than those of prunes, raisins and apricots, respectively. Different from their SPC_W and SPC_A values, the raisins and apricots showed a more balanced distribution of flavonoids in water and alcohol extracts. However, the SFC_W in figs and prunes was 1.8 and 5 fold higher than the SFC_A in these fruits, respectively.

3.2. Antioxidant Capacities of Organic Sun-Dried Fruits

The SAC_T, BAC_T and OAC of sun-dried fruits are given in Table 2. The prunes showed the highest SAC_T that was 1.7, 2.1 and 2.2 fold higher than those of apricots, figs and raisins, respectively. Thus, it is clear that the results of SAC_T showed parallelism with those of SPC_T. However, it is important to note that the SAC_W in different fruits formed 93 to 96% of SAC_T while remaining residual antioxidant capacity was formed by SAC_A. The prunes also had the highest SAC_W that was 1.7 to 2.3 fold higher than those of the other fruits. The apricots showed the second highest SAC_W that was almost 1.2 fold higher than those of figs and raisins. On the other hand, the prunes and apricots showed similar SAC_A values that were 1.3-1.4 fold higher than those of figs and raisins.

The BAC_T of prunes was also 1.6, 1.7 and 2.1 fold higher than those of figs, apricots and raisins, respectively. Thus, the prunes ranked first also in OAC. The apricots and figs showed similar OAC while raisins showed slightly lower OAC than that of these fruits. It is important to note that the BAC_T formed almost 61 to 67 % of OAC in sundried fruits. This result clearly showed that the majority of antioxidant compounds in sun-dried fruits are bound by food hydrocolloids. In the literature different percentages of bound antioxidant capacities were reported for different products. For example, it was reported that in lentils 82-85 % of total antioxidant activity was formed by bound phytochemicals while the percentage of bound antioxidant capacity changes between 25 and 39 % in many other legumes including chickpeas,

yellow beans, green beans, and soybeans (Han & Baik, 2008). A. Serpen *et al.* (2007) reported that 50% of the total antioxidant activity in cereal based food is formed by bound antioxidants. Thus, it appeared that the sun-dried fruit hydrocolloids had a considerable phenolic binding capacity and this could be a limiting factor for the phenolic bioavailability.

Table 2. Total soluble, total bound and overall antioxidant capacities of different organic sun-dried fruits (All units in µmol Trolox/g d.w.).

Product	Soluble			Bound	Overall
	SAC_W	SAC_A	SAC_T	BAC_T	OAC
Raisin	12.1 ± 0.5[c]	0.86 ±0.01[b]	13.0	22.8 ±0.40[c]	36.0
Fig	12.6 ±0.16[c]	0.91 ±0.03[b]	13.5	28.3 ±3.35[b]	42.0
Prune	27.3 ±0.69[a]	1.16 ±0.08[a]	28.5	45.7 ±2.44[a]	74.0
Apricot	15.8 ±0.29[b]	1.20 ±0.16[a]	17.0	27.6 ±1.96[b]	45.0

[a-c] Values followed by different letters are significantly different at $p<0.05$.

3.3. Phenolic and Flavonoid Contents of Organic Sun-Dried Fruits Incorporated with Green Tea Polyphenols

The SPC_T and SFC_T of organic sun-dried raisins, figs, prunes and apricots incorporated with green tea polyphenols are given in Table 3. The SPC_T and SFC_T of green tea polyphenol incorporated sun-dried fruits varied between 2820 and 4215 µg catechin/g (d.w.), and 257 and 658 µg catechin/g (d.w.), respectively. The green tea polyphenol incorporated prunes had the highest SPC_T that was almost 1.5 fold higher than the other green tea polyphenol incorporated fruits. It is also important to note that the SPC_T values of green tea polyphenol incorporated sun-dried fruits except prunes were considerably higher than those of the standard fruits. For example, the SPC_T of raisins was 1.8 fold higher than those of standard raisins while green tea polyphenol incorporated figs and apricots showed almost 1.5 fold higher SPC_T than the standard figs and apricots. In contrast, it is interesting to report that the green tea polyphenol incorporated prunes showed only slightly higher (almost 1.1 fold) SPC_T than that of the standard prunes. On the other hand, in green tea polyphenol incorporated sun-dried fruits, the SPC_W formed 84 to 94% of SPC_T while the remaining phenolic fraction was formed by SPC_A. The green tea polyphenol incorporated prunes showed 1.5 fold higher SPC_W than green tea polyphenol incorporated raisins and apricots, and 1.7 fold higher SPC_W than green tea polyphenol incorporated figs. However, it is important to note that the incorporation of green tea polyphenols caused only a very limited increase in the SPC_W of prunes while it caused 1.3, 1.4 and 1.7 fold increases in SPC_W of figs, apricots and raisins, respectively. The green tea polyphenol incorporated figs showed the highest SPC_A that was 1.3, 1.4 and 2.7 fold higher than those of green tea polyphenol incorporated raisins, apricots and prunes, respectively. The analysis of results also showed that the incorporation of green tea polyphenols caused significant increases in SPC_A fraction of raisins (2.8 fold), apricots (2.3 fold) and figs (2.9 fold) compared to standard fruits. In contrast, the SPC_A of prunes remained almost same by incorporation of green tea polyphenols.

Table 3. Total soluble phenolic and flavonoid contents of different organic sun-dried fruits incorporated with green tea phenolics.

Product	Phenolic content (µg catechin/g d.w.)			Flavonoid content (µg catechin/g d.w.)		
	SPC_W	SPC_A	SPC_T	SFC_W	SFC_A	SFC_T
Raisin	2625 ±40.0[a]	330 ±28.0[b]	2955	196 ±6.6[b]	162 ±16.6[b]	357
Fig	2380 ±94.0[b]	440 ±23.5[a]	2820	362 ±62.4[a]	296 ±19.4[a]	658
Prune	3950 ±26.0[b]	165 ±4.0[c]	4215	403 ±15.1[a]	68 ±2.0[d]	471
Apricot	2670 ±72.5[a]	315 ±7.0[b]	2985	149 ±6.1[b]	108 ±6.2[c]	257

[a-d] Values followed by different letters are significantly different at $p<0.05$.

On the other hand, the results of flavonoid analysis showed that the green tea polyphenol incorporated figs had the highest SFC_T that was almost 1.4, 1.8 and 2.6 fold higher than those of green tea polyphenol incorporated prunes, raisins and apricots, respectively. The incorporation of green tea polyphenol into raisins and apricots caused 1.6 and 2.0-fold increase in their SFC_W, respectively. However, there were only slight increases in SFC_W of figs and prunes by incorporation of green tea polyphenols into these fruits. It is also worth noting that the green tea polyphenol incorporation caused 1.5 and 1.6-fold increase in SFC_A of raisins and figs, respectively, but this process caused slight or inconsiderable increases in SFC_A of green tea incorporated prunes and apricots.

3.4. Antioxidant Capacities of Organic Sun-Dried Fruits Incorporated with Green Tea Polyphenols

The SAC_T, BAC_T and OAC of sun-dried fruits incorporated with green tea polyphenols are given in Table 4. The green tea polyphenol incorporated prunes showed the highest SAC_T that was 1.8 fold higher than those of green tea polyphenol incorporated apricots and figs, and 1.4 fold higher than that of green tea polyphenol incorporated raisins. Thus, the results of SAC_T for green tea polyphenol incorporated fruits showed a high parallelism with their SPC_T results. On the other hand, it is important to note that the green tea polyphenol incorporation increased the SAC_T of apricots, prunes, figs and raisins 1.5, 1.6, 1.9 and 2.6 fold, respectively. The increases in SAC_T of apricots, figs and raisins were expected since the green tea polyphenol incorporation into these fruits caused 1.5 to 1.8-fold increase of SPC_T. The maximal increase of SAC_T by incorporation of green tea polyphenols occurred in raisins that also showed a maximal increase in SPC_T by polyphenol enrichment (1.8 fold). This result was not surprising since absorption of green tea extract into raisins during rehydration is easy due to their small size. However, it is important to note that the high soluble antioxidant activity of raisins is also an indication of low amounts hydrocolloids capable to bind incorporated phenolic compounds. In contrast, it was interesting to report a 1.6-fold increase in SAC_T of prunes that showed an inconsiderable increase in its SPC_T value (1.1 fold) by green tea polyphenol incorporation. This result suggested that the incubation of prunes in green tea infusion caused loss of some water soluble intrinsic prune phenolic compounds from fruits to green tea extract by diffusion. However, it also appeared that almost the same amount of green tea phenolic compound diffused from solution to prunes, and these green tea phenolics got higher antioxidant potential than lost prune phenolics. The SAC_W in different green tea polyphenol incorporated fruits formed 83 to 93% of SAC_T while remaining residual antioxidant capacity was formed by SAC_A. The prunes had the highest SAC_W that was 1.4 to 2.1 fold higher than the other fruits. The raisins showed the second highest SAC_W that is 1.4 fold higher than those of figs and apricots that had similar SAC_W values. The increases in SAC_W of fruits by incorporation of green tea phenolic compounds were similar to those of SAC_T and determined as 1.5, 1.6, 1.7 and 2.5 fold for apricots, prunes, figs and raisins, respectively. Thus, it was clear that 1.3 to 1.7-fold increase in SPC_W of apricots, figs and raisins by incorporation of green tea polyphenols resulted with 1.5 to 2.5-fold increase in their SAC_W values. In contrast, the result of SAC_W for the prunes did not show a high parallelism with results of SAC_T for these fruits. Thus, only a 1.1-fold increase in SPC_W by incorporation of green tea polyphenols caused a 1.6-fold increase in SAC_W of prunes. On the other hand, it is also important to report that the incorporation of green tea polyphenols caused significant increases in SAC_A fraction of all fruits. In green tea polyphenol incorporated apricots, figs and raisins 2.3, 2.8 and 2.9 fold increases in SPC_A by incorporation of green tea polyphenols resulted with 3.0, 4.5 and 3.3 fold increases in SAC_A of these fruits, respectively. In contrast, similar to SAC_T and SAC_W results, a 2.2-fold increase in SAC_A of prunes was observed while there was almost no increase in SPC_A of these fruits. This finding supports our hypothesis that the exchange of green tea phenolic compounds and prune phenolic compounds occurred during rehydration of prunes in green tea extract.

Table 4. Total soluble, total bound and overall antioxidant capacities of different organic sun-dried fruits incorporated with green tea phenolic compounds (All units in μmol Trolox/g d.w.).

Product	Soluble			Bound	Overall
	SAC_W	SAC_A	SAC_T	BAC_T	OAC
Raisin	30.4 ±0.45 [b]	2.84 ±0.09[b]	33.2	30.7 ±1.64[c]	64.0
Fig	21.1 ±0.53[c]	4.1 ±0.19[a]	25.2	39.4 ±3.29[b]	65.0
Prune	43.8 ±1.13[a]	2.55 ±0.17[b]	46.4	68.4 ±4.18[a]	115
Apricot	21.6 ±0.16[c]	3.64 ±0,18[a]	25.2	32.98 ±2.28[bc]	58.0

[a-c] Values followed by different letters are significantly different at $p<0.05$.

The results of bound antioxidant activity measurements showed that the green tea polyphenol incorporated prunes showed 1.7, 2.1 and 2.2 fold higher BAC_T than green tea polyphenol incorporated figs, apricots and raisins, respectively. The increases in BAC_T of fruits by incorporation of green tea polyphenols was between 1.4 and 1.5 fold for raisins, figs and prunes while it was only 1.2 fold for the apricots. These results clearly showed that the incorporation of green tea polyphenols caused a considerably higher increase in soluble antioxidant capacity of prunes, raisins and figs than the bound antioxidant capacity of these fruits. In contrast, the incorporation of green tea polyphenols caused almost similar increases in soluble and bound antioxidant capacities of apricots.

The overall antioxidant capacities (OAC) of fruits clearly showed 1.8 to 2.0 fold higher OAC of green tea polyphenol incorporated prunes than the other green tea polyphenol incorporated fruits that showed similar OAC values. It is important to note that in green tea incorporated fruits the BAC_T formed 48 to 61% of OACs. This result clearly indicated the drop of the percentage of BAC_T in OAC due to the greater increases in soluble antioxidant capacity of fruits than bound antioxidant capacity by incorporation of green tea polyphenols (BAC_T formed 61 to 67 % of OAC in standard sun-dried fruits).

4. CONCLUSION

The results of this study clearly showed the possibility of incorporating green tea polyphenols into sun-dried fruits using controlled rehydration conducted in phenolic rich mediums such as green tea extracts. The procedure applied increased both the soluble phenolic content and soluble antioxidant capacity of raisins, apricots and figs considerably. In prunes that are already a good source of phenolic compounds the rehydration in green tea infusion did not cause a considerable increase in soluble phenolic content, but it increased soluble antioxidant capacity. The use of sun-dried fruits for delivery of green tea phenolic compounds could be an alternative method to increase intake of these invaluable antioxidant compounds and increase functionality of sun-dried fruits which are already accepted as good source of dietary fiber. Further studies are continuing to employ different tea infusions, phenolic extracts and solutions of pure phenolic compounds as alternative to green tea infusions.

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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

Gokhan Dervisoglu: Performed the experimental part and statistical analysis. **Ahmet Yemenicioglu**: supervised the experimental work, wrote and edited the manuscript.

Orcid

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