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International Journal of Secondary Metabolite

International Journal of Secondary Metabolite (IJSM) purposes the publication of articles related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). IJSM is open-access, peer-reviewed academic journal published electronically and quarterly. The journal is the goal to improve the research culture and help knowledge spread rapidly in the academic world by providing a common academic platform in the scope. The journal is published in English.

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

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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

Pharmacological potential of fungal endophytes associated with the genus *Ocimum* L.

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Abstract: Endophytes are a rich source of secondary metabolites such as tannins, phenolics, and alkaloids. Endophytic fungi have potential as antioxidants, antimicrobials, anticancer agents, antidiabetic agents, hepatoprotectants, growth promoters, and immunomodulators. Recent studies have shown that endophytes are a valuable source of undiscovered biomolecules. As a result, endophytic isolates from medicinal plants can be used in the pharmaceutical, industrial, and agricultural sectors. *Ocimum* species, for example, have several medicinal properties and are used in traditional medicine. Fungal endophytes have a strong association with *Ocimum* plants. Previous research has shown that the fungal endophytes of *Ocimum sanctum* produce phytochemicals such as alkaloids, terpenoids, cardiac glycosides, flavonoids, terpenes, and volatile compounds. Additionally, fungal endophytes have a direct impact on the medicinal value of the genus *Ocimum*. This review aimed to discuss the pharmacological properties and diversity of endophytic fungi associated with the genus *Ocimum.*

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1. INTRODUCTION

The genus *Ocimum* come under the family Lamiaceae (mint family). The most commonly known species in this genus include basil, sweet basil, and holy basil. These plants are native to tropical regions of Central Africa and South East Asia, but are now widely cultivated around the world for their culinary and medicinal uses. They are known for their fragrant leaves and can be used in a variety of dishes such as pasta, pizza, and salads. In addition, it has long been used in traditional Chinese and Ayurvedic medicine.

The genus includes approximately sixty-four species, among which *Ocimum basilicum* L. (Rama thulasi), *Ocimum americanum* L. (Lime basil), *Ocimum camporum* Gürke., *Ocimum gratissimum* L. (Clove basil), *Ocimum kilimandscharicum* Gürke., *Ocimum campechianum* Mill., *Ocimum tenuiflorum* L. (*Ocimum sanctum* L. (Holy basil)), etc., are some important species with high medicinal value (Dhama *et al.*, 2021). *Ocimum* is a genus of aromatic perennial herbs and shrubs. The plants in this genus are commonly known as basil. The plants can vary in size, with some species reaching heights of up to 3 meters, while others are more compact and only reach a few inches in height. They have simple, opposite leaves and small, two-lipped, tubular flowers. The flowers are typically white or purple in colour. The stem of

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the plant is usually square-shaped, which is a characteristic of the Lamiaceae family (Nahak *et al.,* 2011).

All species of this genus are highly scented due to the presence of volatile oils such as eugenol (2-Methoxy-4-(prop-2-en-1-yl) phenol), ursolic acid (3-Hydroxyurs-12-en-28-oic acid), carvacrol (2-Methyl-5-(propan-2-yl) phenol), linalool (3,7-Dimethylocta-1,6-dien-3-ol), caryophyllene ((1R,4E,9S)-4,11,11-trimethyl-8-ethylidene bicyclo [7.2.0] undec-4-ene), carvacrol methyl ether, and estragol (1-methoxy-4-(prop-2- en-1-yl) benzene) (Pattanayak *et al.,* 2010). Studies have shown that they possess antioxidant, anti-asthmatic, anti-tussive, anti-malarial, anti-pyretic, anti-inflammatory, anti-diabetic, nematocidal, wound healing, anti-cancerous, cardioprotective and immunomodulatory properties. The essential oil of *Ocimum basilicum* (sweet basil) has been found to have antibacterial, antifungal, and antiviral properties. Additionally, basil has been traditionally used to help with digestion, respiratory issues, and as a pain reliever. The compounds eugenol, linalool, and methyl chavicol present in basil are responsible for its medicinal properties (Pattanayak *et al.,* 2010).

Endophytes are microorganisms, such as bacteria and fungi, that live within the tissues of plants, often without causing any harm to the host plant. These microorganisms can be found throughout the entire plant, including the leaves, stems, roots, and seeds. It can be defined as "*all organisms that asymptomatically colonize the living internal tissues of their hosts during a variable period of time*" (Stone *et al*., 2004). Endophytes are found in many different types of plants, including grasses, shrubs, trees, and medicinal plants. Endophytes are also known to have a mutualistic relationship with plants, where both the endophyte and the host plant benefit from each other's presence. Regardless of the plant organ affected, endophytes and hosts interact in a balanced antagonistic manner. The fungus is always at least relatively virulent, which facilitates infection, whereas the plant host's defence inhibits the growth of fungal invaders as well as diseases (Schulz & Boyle, 2005).

Endophytes have been found to have a variety of beneficial effects on their host plants, such as increased tolerance to abiotic stress (e.g., drought, heavy metals, salinity), improved nutrient acquisition, and increased resistance to pathogens. Some endophytes can also produce secondary metabolites that have medicinal properties (Zhang *et al.,* 2006; Sudha *et al.,* 2016). Research on endophytes is an active area of study, and scientists are still working to understand the full range of their effects on plants and their potential uses in agriculture and medicinal sectors. Studies have shown that *Ocimum* plants can harbour a diverse community of fungal endophytes, including species from the genera *Fusarium, Penicillium*, and *Aspergillus* (Table 1). Some of these fungal endophytes have been found to have beneficial effects on *Ocimum* plants. Studies have reported that fungal endophytes can enhance the secondary metabolism of *Ocimum* plants, which can lead to increase in the production of essential oils and flavonoids (Chowdhary & Kaushik, 2015). It is believed that the presence of associated fungal endophytes directly influences the medicinal properties of the genus *Ocimum*.

This review paper tries to discuss the pharmacological potential and diversity of fungal endophytes associated with the genus *Ocimum.*

2. DIVERSITY

Endophytes have a cosmopolitan distribution. They are a highly diverse group of organisms and reside in almost all tissues of the host plant, like roots, stems, leaves, petioles, flowers, barks, etc. Their diversity depends on certain factors like age of the host plant, atmospheric humidity, and average temperature (Chaeprasert *et al.,* 2010). The genus *Ocimum* were associated with diverse group endophytic fungi (Table 1). Among the various tissue, leaves are more species rich due to their thin cuticle, which aids easy penetration, and the presence of photosynthetic areas with loosely arranged cells. From various age-grouped leaves of *O.*

tenuiflorum, 148 endophytes were isolated (Taufiq & Darah, 2018)., and the highest number of isolates was obtained from old leaves with 39.86%, followed by senescent leaves with 25.00%, mature ones with 22.30%, and younger leaves with 12.48% of the isolation rate.

Table 1. Endophytic Fungal species diversity of the genus *Ocimum*

Name of the host	Fungal endophytes	Reference
Ocimum tenuiflorum	*Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara., *Pleosporales* sp., *Phomopsis archeri* B. Sutton., *Colletotrichum gloeosporioides* (Penz.) Sacc., *Fusarium* sp., *Nigrospora* state of *khuskia oryzae* H. J. Hudson., *Penicillum* sp., *Aspergillus flavus* Link., *Nigrospora sphaerica* (Sacc.) Mason., *Alternaria raphani* J. W. Groves and Skolko., *Curvularia borreriae* (Viegas) M.B. Ellis., *Cladosporium sphaerospermum* Penz., *Phoma glomerata* Wollenw and Hochapf., *Alternaria alternata* (Fr.) Keissl., *Alternaria tenuissima* Samuel Paul Wiltshire., *Aspergillus niger* van Tieghem., *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker., *Chaetomium coarctatum* Sergeeva., *Curvularia lunata* Boedijn., *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg., *Fusarium solani* Mart., *Fusarium verticillioides* (Sacc.) Nirenberg., *Hypocrea* sp., *Hypoxylon* sp., *Macrophomina phaseolina* (Tassi) Goid., *Meyerozyma guilliermondii* (Wick.) Kurtzman & M. Suzuki., *Penicillium crustosum* Thom., *Rhizoctonia bataticola* (Tassi) Goid.	(Bodhankar, 2014; Chowdhary & Kaushik 2015; Shukla *et al.,* 2012)
Ocimum basilicum	*Phoma eupyrena* (Sacc.) Valenz.-Lopez, Crous, Stchigel, Guarro & J.F. Cano., *Emericella nidulans* G.Winter., *Chaetomium olivaceum* Cooke & Ellis., *Chaetomium globosum* Kunze ex Fries., *Nigrospora oryzae* (Berk. & Broome) Petch., *Alternaria citri* Ellis & N. Pierce, *Alternaria alternata* (Fr.) Keissl.	(Shekhawat & Shah 2013)

3. BIOACTIVE COMPOUNDS

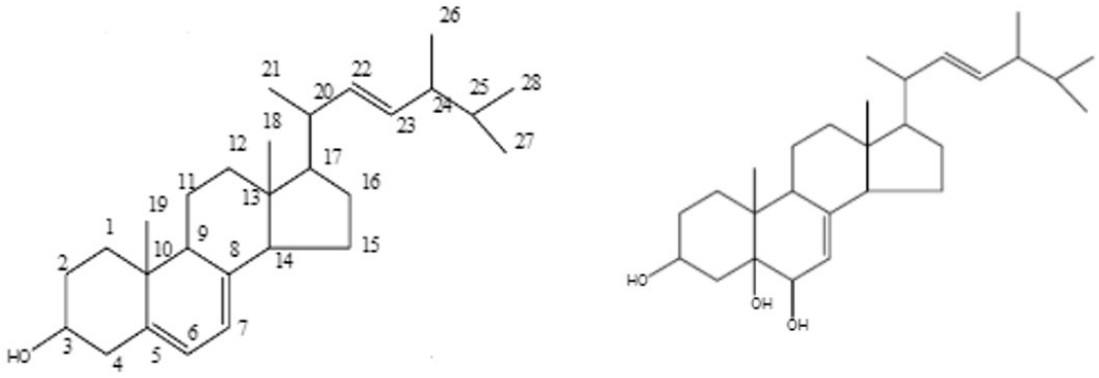
Preliminary phytochemical screening of endophytic fungal crude extract from *O. basilicum* revealed the presence of terpenes, volatile compounds, fatty acids, aliphatic constituents, alkaloids, terpenoids, cardiac glycosides, and flavonoids (Shekhawat & Shah, 2013). Similarly, Gas Chromatography Mass Spectroscopy (GC-MS) chromatogram analysis of the isolate *Macrophomina phaseolina* isolated from the leaves of *O. tenuiflorum* showed the presence of 2H-Pyran-2-one, 5, 6-dihydro-6-pentyl (RT 30.156), hexadecanoic acid (RT 53.017), linoleic acid (RT 64.986), and 10-Octadeconic acid (RT 65.265) (Chowdhary & Kaushik, 2015). Among them, the compound 2H-pyran-2-one is the first report from an endophyte and it exhibited anti-phytopathogenic activity with IC_{50} value of 1.002 and 0.662, respectively, against *Sclerotinia sclerotiorum* (Chowdhary & Kaushik, 2015).

Studies by Mohammad *et al.,* (2015) on the crude extract of endophytes isolated from *O.tenuiflorum,* elucidated the structure of linoleic acid, R (-)-glycerol monolinoleate, 9, 10, 11,-trihydroxy-(12Z)-12-octadecenoic acid, ergosterol, ergosterol peroxide, 1,8-O-dimethylaverantin, coriloxin, and a new natural product named as sactumol. Studies of Gangadevi & Muthumary, (2007) showed that leaf endophyte *Phyllosticta* (sp.6) is a potential producer of taxol in artificial culture media. Similarly, *Nigrospora* sp. isolated from *O.*

basilicum also produces biomolecules such as 5E, 9E-farnesyl acetone, columellarin, totarene, laurenan-2-one, and 8S, 13-cedranediol (Haque *et al.,* 2005).

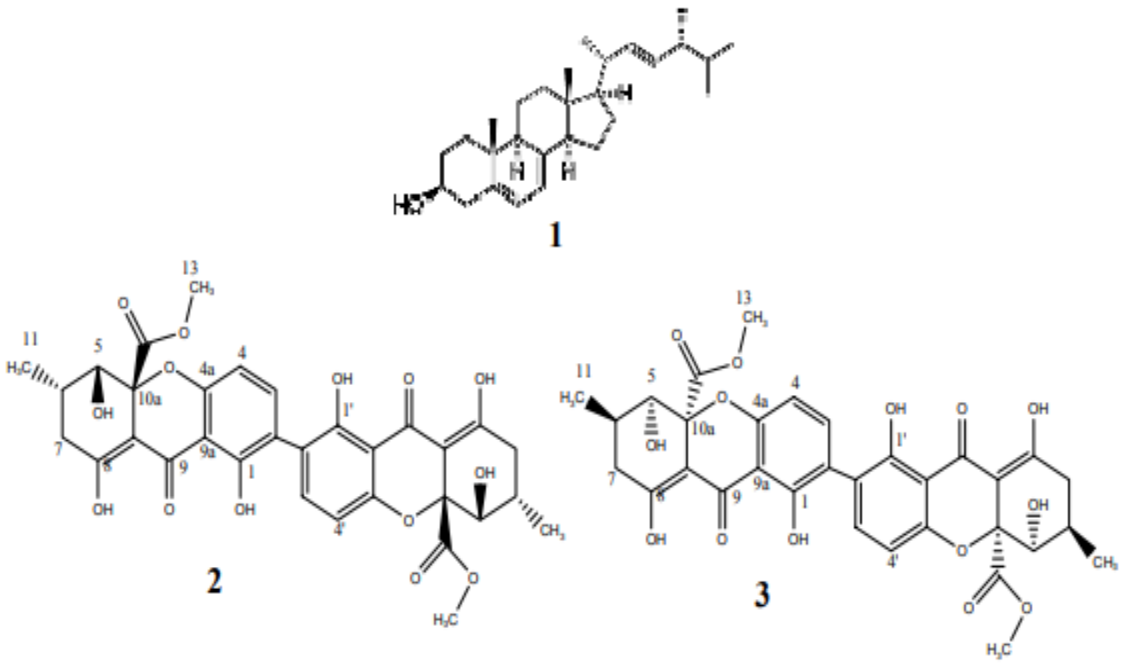
The endophytic fungal isolate *Penicillium citrinum* from *O. tenuiflorum* was a significant source of bioactive polyketides and alkaloids. The structural elucidation of biomolecules isolated from them reported a new compound (compound 6) (Lai *et al.,* 2013). Phytochemical screening of methanolic crude extract of endophytes from *O. basilicum* has revealed the presence of alkaloids, steroids, terpenoids, and saponins (Abdel-Rahman *et al.,* 2019). Studies of Haque *et al.,* (2005) were able to isolate two sterol compounds from endophytic extract by using silica gel column chromatography with different proportions of mobile phase (ethyl acetate, hexane, and methanol), and their purification resulted two compounds as ergosterol and cerevesterol (Figure 1). Similarly, studies of Shoeb *et al.,* (2013), with HR-ESIMS spectrum analysis of ethyl acetate extract of fungal endophyte revealed the presence of three bioactive compounds such as ergosterol (1), secalonic acid A (2), and secalonic acid D (3) (Figure 2).

Figure 1. Ergosterol & Cerevesterol



Ergosterol Cerevesterol

Figure 2. (1) Ergosterol, (2) Secalonic acid A & (3) Secalonic acid D



4. ANTI CANCER ACTIVITY

Silver nanoparticles produced from *Exserhohilum rostrata,* an endophyte from the leaves of *O. sanctum.* By evaluating the anti-cancer potential of this silver nanoparticles showed effective inhibition activity against breast cancer cells (Bagur *et al.,* 2020). Shoeb *et al.,* (2013) evaluated the cytotoxicity of the biomolecules from the endophytes isolated from *O. basilicum* by using MTT assay against a human pancreatic cancer cell line and found that, among the identified biomolecules, secalonic acid A and D exhibited significant anti- cancer activity, with an IC_{50} value of 7.3 and 1.6 mg, respectively Shoeb *et al.,* (2013). Likewise, two compounds (compounds 12 and 17) obtained from the extract of *Penicillium citrinum*, exhibited significant activity against the marine lymphoma cell line L5178Y, with an IC_{50} value of 1.0 and.78 mg/ml, respectively (Lai *et al.,* 2013).

5. HEPATOPROTECTIVE ACTIVITY

The hepatoprotective activities of root fungal endophytic fractions (TRF) 1 (identified as *Paecilomyces variotii*) from *O. sanctum* were evaluated through CCL4 (carbon tetrachloride) induced hepatotoxicity *in vivo* in rats. The results have revealed that the fungal culture filtrate is more potent and significantly reverse the action of carbon tetrachloride induced hepatotoxicity by the restoration of serum AST, ALT, ALP, bilirubin, triglycerides, and protein levels to normal values. It is an indication of stabilization of the plasma membrane as well as repair of hepatic tissue damage caused by CCl4 (Shukla *et al.,* 2012).

6. IMMUNOMODULATORY ACTIVITY

Fungal endophytes from the roots of *O. sanctum* (designated as TRF-1, TRF-2, TRF-3, and TRF-6) were subjected to *in vitro* immunomodulatory activity. The effect of culture extract on the functions of human polymorphonuclear (PMN) cells such as phagocytosis, intracellular killing activity of *Candida albicans*, chemotaxis, and reduction of nitro blue tetrazolium (NBT) dye were evaluated (Madagundi *et al.,* 2013). The result showed that a dose-dependent decrease in the neutrophil was observed with a maximum reduction of 89.78% for TRF-3 and 74.75% for TRF-6, respectively, in the NBT assay. The mean particle amount of phagocytosis of killed *C. albicans* was found to be 7-8 for both TRF-3 and TRF-6, respectively, at 100 g/mL as compared to standard. In the chemotaxis assay, treatment with TRF-3 and TRF-6 showed a maximum number of neutrophils at 100 g/mL, which was comparable with the standard (Madagundi *et al.,* 2013).

7. GROWTH PROMOTING ACTIVITY

Serendipita indica, isolated from the leaves of *O. basilicum,* was tested for its growth enhancing ability on heavy metal contaminated soil. The greenhouse pot experiments under controlled conditions revealed that the culture filtrate treated plants have increased root and shoot dry weight and a decreased concentration of lead and copper in their shoots, but they do not enhance the essential oil content (Sabra *et al.,* 2018). Similarly, Shebany *et al.,* (2013) evaluate the effect of endophytes on the growth and essential oil content of *O. basilicum*. The culture filtrate of *Chaetomium globosum* (isolate no. 1)*, C. globosum* (isolate no. 2)*, Nigrospora oryzae, Alternaria citri,* and *A. alternata* were used for the study. Application of culture filtrate to the soil, significantly increasing the stem diameter, number of leaves, fresh and dry weight of the plant, and concentration of secondary metabolites such as methyl chavicol, limonene, and total oil content as compared to untreated control plants Shebany *et al.,* (2013).

8. ENZYME ACTIVITY

A study by Kumar *et al.,* (2014) on the endophytes of *O. sanctum* found that 50% of the isolates produced amylase and protease, and 27.5% yielded tyrosinase. Zaidi *et al.,* (2013) evaluated

the tyrosinase activity, both qualitatively and quantitatively and found that *Aspergillus* sp. and *Penicillium* sp. (TYR-26, TYR-32, and TYR-38) showed higher tyrosinase activities of 2.8, 3.2, and 2.43 U/ml, respectively. Studies by Shekhawat & Shah, (2013), on extracellular enzyme assays showed that cellulase activity was present with 88.8% of total isolates of endophyte, 77.7% for pectinase, 55.5% for amylase, and 77.7% for tyrosinase.

9. ENZYME INHIBITORS

Culture filtrate of *Phoma* sp., *Eupyrena* sp., *Emericella nidulans* var. *lata,* and *Chaetomium olivaccium* isolated from *O. basilicum* were tested for their anti-amylase activity; all extracts inhibited ($p < 0.01$) alpha amylase. The extracts probably non-competitively bind to the active site of the enzyme. The highest activity was found in *Phoma* with an IC_{50} of 0.108, followed by *Emericella* (IC50 of 0.056) and *Chaetomium* (IC50 of 0.046) at 50g/ml. At 25g/ml, *Phoma* had the highest inhibitory activity with an IC_{50} of 0.083 g/ml (Abdel-Rahman *et al.,* 2019). Likewise, studies of Madagundi *et al.,* (2013) have shown that fungal endophytes of *O. sanctum* were able to inhibit alpha-glucosidase and alpha-amylase. *In vitro* analysis of ethyl acetate extracts of *A. tenuissima* (POST34) and *Trichoderma* sp. (POST047) have demonstrated inhibition against pancreatic-amylase enzyme with IC_{50} values of 27.34 and 40.73 g/mL, respectively Madagundi *et al.,* (2013).

10. PRODUCTION OF SILVER NANOPARTICLES

Extract of *Exserohilum rostra* from *O. tenuiflorum* leaf is used for the preparation of AgNPs and they are evaluated for activities like antibacterial, anti-inflammatory, antioxidant, and anti-proliferative activity in breast cancer cells. The result showed that biomolecules of endophytic extracts capped with AgNPs have a significant level of activity (Bagur *et al.,* 2020).

11. ANTI BIOFILM ACTIVITY

Ethyl acetate extracts of *Lasiodiplodia pseudotheobromae* IBRL OS 64 were evaluated for their anti-biofilm activity against *Streptococcus mutans*. A Congo red agar and a semi-quantitative adherence assay were performed to determine biofilm formation and anti-biofilm activity, respectively. The results have shown that they have good anti-biofilm activity with 89.23% of inhibition for the initial biofilm and 54.14% of inhibition for the preformed biofilm of *S. mutans*. The extracts significantly eliminate the extracellular polysaccharide matrices and hinder the attachment of bacterial cells for biofilm formation (Jalil & Ibrahim, 2021). Similarly, (Bagur *et al.,* 2020) showed the nanoparticle (AgNPs) synthesized from the endophytic fungal extract have the ability of biofilm inhibition against *P. aeruginosa* and *S. aureus*.

12. ANTIOXIDANT ACTIVITY

The ethyl acetate extract of *O. sanctum* root endophyte was subjected to *in vitro* free radical scavenging (2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, hydroxyl radical assay, and reducing power assay, and the isolates TRF-3 and TRF-6 had IC50 values of 271.74 g/mL and 140.54 g/mL for the DPPH (Madagundi *et al*., 2013). In a similar study, it was observed that TRF1 (*Paecilomyces variotii*) had the highest IC50 value at 71.83 g/mL for DPPH and 110.85 g/mL for the hydroxyl radical, respectively (Shukla *et al*., 2012). Fungal endophytes isolated from the roots, stems, and leaves of *Ocimum basilicum* were evaluated for their antioxidant activity and quantitatively determined. The results showed that the IC50 values for DPPH radical scavenging assay were in the following order: *Phoma* (PHE) > *Emericella* (EME) > *Chaetomium* (CHE). The values were 63.3%, 56.2%, and 26.8% at 100 g/mL, respectively. The scavenging activity for hydrogen peroxide of various extracts was in the following order: PHE > CHE > EME, respectively (Abdel-Rahman *et al*., 2019).

13. ANTIMICROBIAL ACTIVITIES

Fungal endophytes were isolated from the leaves of *Ocimum basilicum* and tested against nine human pathogens. The study found that *Nigrospora* (MFLUCC16-0605) exhibited broad-spectrum activity, with minimum inhibitory concentration (MIC) values ranging from 7.81 to 250 g/mL (Atiphasaworn *et al.,* 2017). Similarly, hexane, methanol, and ethyl acetate extracts of *N. oryzae* showed promising levels of activity against *E. coli*, *K. pneumoniae*,*S. aureus*, *S. typhimurium*, *B. cereus*, and *B. subtilis* (Bodhankar, 2014). Compared to the younger leaves of *O. sanctum,* isolates obtained from older leaves have significant antibacterial activity. All the tested isolates showed enhanced activity against at least one of the tested pathogens, and the activity was enhanced after adding water extract of the host plant into the culture medium (Taufiq & Darah, 2019). The extracts of *L. pseudotheobromae* IBRL OS-64 from *O. sanctum* were tested against food-borne bacteria and found that the ethyl acetate extract had antibacterial activity towards all test bacteria with an inhibition zone ranging from 15.0 mm to 26.0 mm (Taufiq & Darah, 2019). Dichloromethane extract of fungal endophytes from the mature leaves of *Ocimum kilimandscharicum* showed significant anti-bacterial activity (Ismail, 2015). Endophytes of *Ocimum africanum* effectively inhibited methicillin-resistant *S. aureus*, *K. pneumoniae*, and *Shigella boydii* (Nur Amalina, 2015). The dichloromethane and methanol extract of the fungal isolate of *Ocimum basilicum* var. *thyrsiflora* leaves displayed the highest antimicrobial activity against the selected bacteria (Omar, 2015).

Studies of Shekhawat & Shah, (2013) evaluated the anti -bacterial potential of five fungal endophytes associated with *Ocimum sanctum* against *E coli*, *Bs megaterium*, *Paeruginosa*, *S marsences*, *B. subtilis*, *S. aureus*, *M.luteus* and *S. typhi*. Among them, the fungal isolate (F GTS 2) significantly inhibited *P. aeruginosa*, and three fungal isolates (F GTS 2, F GTL4, and F GTS 5) significantly inhibited *M. luteus* (Shekhawat & Shah, 2013).

Fungal endophytes isolated from *Ocimum sanctum* were evaluated against *Rhizoctonia solani*, *Fusarium oxysporum*, *Colletotrichum falcatum*, and *Helminthosporium maydis*. A total of nine fungi were isolated, but none of them showed inhibition of mycelial growth (Yadav *et al.,* 2015).

14. CONCLUSION

Research on endophytes is important because these microorganisms can have a wide range of beneficial effects on their host plants, including increased tolerance to environmental stress, disease resistance, and improved nutrient acquisition. Additionally, endophytes have potential for use in agricultural and biotechnology applications, such as the development of biopesticides. Overall, understanding the interactions between endophytes and plants can lead to improved crop yields and sustainable agricultural practices. Endophytes are a rich source of bioactive molecules such as antibiotics, enzymes, and secondary metabolites. These molecules have a wide range of potential applications, including in medicine, agriculture, and industry. Studies showed that numerous biologically active substances can be produced by endophytes of the genus *Ocimum*. They share structural and functional similarities with chemicals made by *Ocimum* plants. Exploiting the pharmacological potential of endophytes will assist the pharmaceutical sector in developing novel, affordable treatments against devastating diseases.

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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

Manikandan Karthika: Wrote the first draft. **Avanoor Ramanathan Rasmi:** Supervision, validation, and correction of the final draft.

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Research Article

Biological activities of different plant species belonging to the Asteraceae family

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Abstract: *Achillea biebersteinii* and *Anthemis tinctoria*, which are widely distributed species of the Asteraceae family, are used in folk medicine in the form of herbal tea or extract in the treatment of many diseases. The aim of this study was to investigate the chemical content, antimicrobial, antioxidant, enzyme inhibitor activities and cytotoxic effects of 80% ethanol extract of these two species and make a comparative analysis. In accordance with the data obtained, the major component of *A. biebersteinii* was determined as Cyclododecane (14.47%), while that of *A. tinctoria* was determined as Phytol (23.15%). *A. biebersteinii*, which showed moderate activity in terms of antimicrobial activity, produced more active inhibition than *A. tinctoria* did. Both plants showed high levels of antioxidant activity. The total phenol and total flavonoid contents of *A. tinctoria* were higher than those of *A. biebersteinii*. It was determined that there was no significant activity when the extracts were compared with galanthamine, which is the reference drug in terms of enzyme inhibitory activity. When the *in vitro* anticancer activity of human breast cancer cell line was examined, it was determined that *A. tinctoria* had a cytotoxic effect at high concentrations (IC_{50};0.82mg/mL), and *A. biebersteinii* showed strong cytotoxicity at all concentrations (IC_{50};<0.0625mg/mL). These two plants of the same family were evaluated in terms of many different biological parameters and it was revealed that *A. biebersteinii* was more active than *A. tinctoria*. However, in vivo studies are needed to determine whether these plants can be used as phytotherapeutic agents.

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1. INTRODUCTION

The Asteraceae family has a wide distribution, especially in the Caucasus, Central Asia and Europe, with 1100 genus and approximately 25000 species worldwide. In Turkey, although most are endemic, there are 152 genus and 1230 species of this family (Yıldırımlı, 1999). Many species of this family are both edible and can be used as medicinal plants, as they generally

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have some content rich in secondary metabolites such as essential oil compounds, sesquiterpenes and flavonoids. Due to this richness of content, it is used in medicinal herbal teas and standardized extracts as an antitussive, anti-inflammatory, and sedative, as well as in skin care and cosmetic products (Li *et al*., 2013; Butala *et al*., 2021). Furthermore, in vitro studies have reported that species belonging to this family are potential therapeutic agents for various tumorigenesis (El Omari *et al*., 2021).

The antimicrobial activity of edible plants may play an active role in reducing antibiotic use or inhibiting specific pathogens (Dzoyem *et al*.,2013; Panda *et al*.,2019). Oxidative stress has a complex effect on metabolism, and there can be said to be multiple variables that affect physiological reactions in this process. The type of oxidant, density, and interaction with antioxidant compounds may cause different results on metabolic activities (Baroni *et al*., 2021). Antioxidants acting in the defense mechanism of cells may not be sufficient to suppress free radicals, and when a pathological result occurs, exogenous antioxidants may have a positive effect on the healing process. In such a case, antioxidant-rich medicinal plants can be used to strengthen metabolic defense (Li *et al*., 2014; Xu *et al*., 2017; Yu *et al*., 2021).

The two best-known genera of the Asteraceae family are *Achillea* and *Anthemis*. Many species in these two genera are actively used in folk medicine (Nemeth & Bernath, 2008). In particular, the antioxidant and antimicrobial effects of different species belonging to these genera have been shown in many studies (Candan *et al*., 2003; Kotan *et al*., 2010; Varasteh-Kojourian *et al*., 2017; Elshamy *et al*., 2021; Hoi *et al*.,2021).) In addition, phytochemical studies have reported that these plants have antioxidant, antiproliferative, antidiabetic, antiprotozoal, and antispasmodic potential (De Mieri *et al*., 2017; Şabanoğlu *et al*., 2019).

In this study, chemical compound analysis, antioxidant, antimicrobial activities and antiproliferative effects of *Achillea biebersteinii* and *Anthemis tinctoria* from the Asteraceae family growing in the flora of Turkey were investigated. It is expected that the obtained data will add positive value to phytotherapeutic studies.

2. MATERIAL and METHODS

2.1. Plant Material and Extract Preparation

The aerial parts of the plants in full flowering periods were collected. *A. tinctoria* was collected from Yozgat-Akdağmadeni (39°39`N; 35°,52` E, 866 m) and *A. biebersteinii* was collected from Yozgat-Gedikhasanlı (39°,35` N; 35°,9` E, 1128 m) in June 2017. The collected fresh aerial parts were dried at room temperature. The plants were identified in Yozgat Bozok University Biology Department.

After the collected plant samples (leaves) were dried and ground, 10 grams of samples were taken and dissolved in 80% ethanol. GC-MS was utilized to determine the ingredients and relative percentages of the extracts that were filter-dried in a rotary evaporator (Sacchetti *et al*., 2005). Helium gas was used as the carrier gas at a constant flow rate of 1.5 ml per minute. In splitless mode, the injection volume of 1 µl was designed to be 5 per minute between 80-300 and was set at 300°C for 2 minutes after the run. Total running time was 1 hour (Eruygur & Dural, 2019). The chemical content of the extract acquired from the dried leaves was researched through different libraries (W9N11.L, NIST05a.L and wiley7n.I).

2.2. Specification of Total Phenolic Content

The total phenol content of the plant extracts was determined through reaction with Folin-Ciocalteu (F-C) reagent. Extracts diluted first with DMSO were mixed with distilled water and diluted F-C reagent. After waiting for 5 minutes, 7.5% Na_2CO_3 was suffixed and incubated for 1 hour and finally absorbance was measured at 650 nm. DMSO (blank) and Gallic acid in DMSO (reference) were also run in parallel.

2.3. Evaluation of Total Flavonoid Content

The total flavonoid content in the plant extracts was defined through the aluminum chloride colorimetric assay. It was calibrated by preparing serial dilution solutions. The reagent (150 µL, 0.3 mg/mL) prepared with ethanol was mixed with 2% $AlCl_3$ on a microplate. The absorbance value of the solution, which was kept at 22°C for 15 min, was measured at 435 nm. Then, the total flavonoid contents of the extracts were expressed as mg of quercetin equivalent on their dry weight.

2.4. DPPH Assay

The antioxidant activity of the extracts was expressed as a percentage and the procedure was performed in accordance with the DPPH free radical assay as stated by Eruygur *et al.* (2019). The DPPH solution was freshly prepared by dissolving in ethanol. 20 µL of the plant extracts dissolved in DMSO were mixed with 180 µL of DPPH solution (40 µg/mL) in a 96-well plate. After the well plates were left in the dark for fifteen minutes, absorbance levels were measured at 540 nm on a spectrophotometer. DMSO and gallic acid were accepted as control and standard, respectively. The experiments were repeated three times and the results were evaluated by calculating the standard deviation (SD).

2.5. ABTS Radical Scavenging Assay

Test samples and ABTS radical stock solution were prepared in accordance with the DPPH method and diluted (7 mM ABTS, 140 mM potassium persulfate) just before analysis, then stored at room temperature. The ABTS working solution was prepared fresh by diluting with ethanol, the absorbance value was measured at 734 nm and the value was taken as 0.70 ± 0.02. On a microplate, 50 µL of sample solution at a concentration of 0-1 mg/mL was mixed with 100 µL of ABTS working solution, the mixture was incubated for 10 minutes at room temperature, and then the absorbance was read at 734 nm. ABTS scavenging activity was evaluated using gallic acid as antioxidant standards.

2.6. Antineurodegenerative Activities

Acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and also tyrosinase inhibitory activity assays were performed according to the protocol of Eruygur *et al.* (2019) using a microtiter plate according to the spectrophotometric method. The applied extract concentration was 2 mg/ml and galantamine and kojic acid were accepted as positive controls. The absorbance was determined with an Epoch microplate reader. The results are shown as percentage inhibition of the samples compared to the controls.

2.7. Antimicrobial Activity

Using the microdilution method of Eloff (1998), the Minimum Inhibitory Concentration (MIC) values of the ethanol extracts obtained from the plants used in the study on predetermined bacteria and fungi were determined. *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus faecalis* (ATCC 29212) strains were used as microbial agents in this study. The extract was dissolved with 8% DMSO. Then, 10 µl of sample was added to the first line of the microtiter plate diluted with 90 µl of broth. In the second line, 50 µL of sample was added and two-fold serial dilution was made with the broth. The concentration of the plant extracts in the well after the application was 5.00-0.002 mg/ml. The final inoculum size was 0.5-2.5×103 CFU/mL in Candida and 5×105 CFU/mL in bacteria per well. Candida culture was diluted with Mueller Hinton Broth (Accumix®) and bacterial culture was diluted with Sabouraud Dextrose Broth (Himedia ME033). 50 µL of fungal and bacterial suspension were added to the prepared samples and incubated for 16-24 hours at 35°C and 37°C, respectively. To indicate growth, 50 µL (2 mg/mL) of 2,3,5-Triphenyltetrazolium chloride (TTC) (Merck, Germany) was added to

each well. Microtiter plates were also incubated for 2 hours at 37°C. The decrease in the intensity of the red color of formazan at the end of this period was accepted as the MIC value. The experiment was repeated in duplicate and the standard deviation was recorded as zero.

2.8. Cell Viability Assay

Human breast cancer cell line, MDA-MB-231 and mouse fibroblast cell line, L929 were obtained from the ATCC (USA) and maintained in DMEM supplemented with 100 U/mL penicillin, 10% fetal bovine serum and 100 µg/mL streptomycin. The cell viability of the 80% ethanol extracts of *A. bieberstenii* and *A. tinctoria* was assessed using the XTT assay. In short, exponentially growing cells were seeded in 96-well plates and cultured until 50% confluence. Various concentrations of the plant extracts were then added and the final concentration in each well was 0.0625, 0.125, 0.25, 0.5, 1 mg/mL, respectively for 24 hours. Untreated and 0.5%-DMSO-treated cells were designated as negative and solvent control, respectively. At the end of exposure, the detection of viable cells was performed by adding 50 µL of XTT labeling mix to each well and re-incubating at 37°C for 4 hours. The absorbance of each well was measured by a microplate reader (Thermo, Germany) at 450 nm versus the control. The cell proliferation was calculated as % relative to the control (100% viability).

2.9. Statistical Analysis

The results of the biological activity analysis repeated three times were expressed as mean ± standard deviation values. Statistical evaluation of the obtained data was performed with Graphpad 6.0 software.

3. RESULTS and DISCUSSION

3.1. GC-MS Analysis of the Extracts

In this study, the chemical components of 80% ethanol extracts of *A. biebersteinii* and *A.tinctoria* were evaluated using GS-MS (Table 1).

Table 1. Chemical components of 80% ethanol extracts of *A. biebersteinii and A. tinctoria.*

Chemical Components	RT	Relative Content (%)	
		A.biebersteinii	*A. tinctoria*
Pyrrolidine, N-(3-methyl-3-butenyl)	12.917	1.27	
1,4:3,6-Dianhydro-.alpha.-d-glucopyranose	18.673		1.31
1-Dodecanol (CAS)	28.258	3.87	2.34
Phenol, 2,4-bis(1,1-dimethylethyl)	29.379		1.44
(-)-Caryophyllene oxide	31.222	0.96	
Maltoxazine	32.618	1.72	
Cyclododecane	33.465	14.47	7.08
(-)-Loliolide	35.130	2.29	1.25
Quinoline, 3-(methylthio)-	36.228		1.40
Hexadecanoic acid (CAS)	38.397		2.00
Hexadecanoic acid, ethyl ester (CA	38.844	6.12	4.43
Phytol (CAS)	40.732	4.72	23.15
9-Octadecenoic acid, (E)-	41.224		11.38
Ethyl linoleate	41.447	3.49	
Ethyl Oleate	41.544		3.82
Isosteviol methyl ester	45.945	3.30	2.90
Methyl steviol	46.048	11.86	3.17
TOTAL		54.07	65.67

According to the data obtained, "Cyclododecane" was the main component for *A.biebersteinii* at 14.47%, followed by "Methyl steviol" at 11.86%. The main component of *A. tinctoria* was determined as "Phytol" at 23.15%, followed by "9-Octadecenoic acid" at 11.38%. It was observed that the number of components determined at the beginning of flowering was higher than the value obtained from the plants harvested during the full flowering period. Sevindik *et al*. (2018) determined the main components of the same plant to be 1,8-cineole (20.36%) and cyclohexanone (8.39%). Emir & Emir (2020) identified morin and quercetin (1598.4 µg/g, 1416.0 µg/g, respectively) as the main components of flavonoids from *A. tinctoria*. As the phenolic contents may vary when the plants are collected from different localities, the seasonal and growing conditions should be evaluated.

3.2. Antioxidant Activity

The studied extracts exhibited ABTS scavenging activity near to that of the positive control gallic acid and it was dose-dependent in all species (Figure 1). The IC_{50} value calculated for the extracts was 18.06 ± 0.01 µg/mL for *A. biebersteinii* and 6.37 ± 0.01 µg/mL for *A. tinctoria.* As seen in Figure 1, ABTS radical scavenging activity of the extracts was close to that of the reference drug gallic acid (IC_{50}: 0.39 ± 0.01 µg/mL). The DPPH radical scavenging activity IC_{50} value calculated for the extracts was 502.9 ± 0.009 µg/mL for *A. biebersteinii* and 399.04 ± 0.02 µg/mL for *A. Tinctoria* which was lower than that of gallic acid (IC_{50}: 29.11 ± 1.76 µg/mL) (Figure 2). In a previous study, the leaf and plant methanol extracts of *Achillea sivasica* showed strong activity with IC_{50} values of 0.12 µg/mL and 0.22 µg/mL when tested for DPPH free radical scavenging activity (Haliloglu *et al*., 2017). In another study, the *Achillea millefolium* extracts represented moderate DPPH radical scavenging activities with SC_{50} values of 0.266 ± 0.003 mg/mL for methanol extract and 0.495±0.005 mg/mL for ethanol extract (Barut *et al*., 2017).

The antioxidant activity values of 80% ethanol extracts of *A. biebersteinii, A. tinctoria* were analyzed and evaluated using DPPH and ABTS methods. According to the data obtained, it can be said that *A.biebersteinii* and *A.tinctoria* (IC_{50} value: 18.06 ± 0.008 µg/mL, IC_{50} value: 6.37 ± 0.009 µg/mL, respectively) have a high level of antioxidant activity, especially for ABTS radical scavenging activity. According to the DPPH test, when the extract values and gallic acid values were compared, the antioxidant activity values were seen to be moderate. The antioxidant activity of *A. tinctoria* with DPPH and CUPRAC methods was investigated by Emir & Emir (2020) and IC_{50} values were found to be 302.18 ± 2.74 and 514.62 ± 3.82 mg TE/g, respectively. Varasteh-kojourian *et al*. (2017) investigated the antioxidant activity of *A. biebersteinii* by preparing ethanol and methanol extracts from different parts (inflorescence, stem, leaves and aerial parts) of the plant, and reported that the methanol leaf extract of the plant was significantly higher (0.0276 ± 0.003 mg/ml for DPPH, 0.16 ± 0.016 mg/ml for BCB and 13.96 ± 0.26 mg/ml for TBARS IC_{50}s) than that of the other extracts. Sabanoğlu *et al*. (2019) compared two different *Achillea* species and found that the radical scavenging activity of *A. biebersteinii* was considerably higher than that of *A.wilhelmsii*. The results of the different studies demonstrated that both species, especially the methanol extract, show high antioxidant activity. The current study with ethanol extract of the same species showed similar results to previous studies and showed high radical scavenging activity.

Figure 1. ABTS radical scavenging activity of *A. biebersteinii* and *A. tinctoria* ethanol extracts.

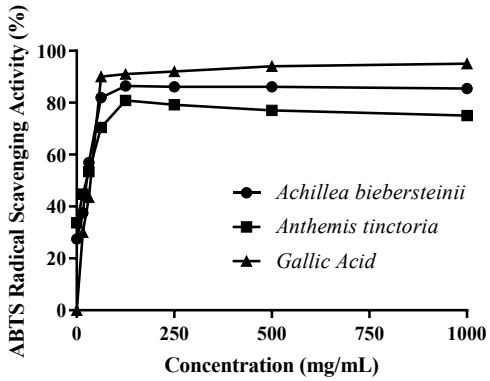


Figure 2. DPPH radical scavenging activity of *A. biebersteinii* and *A. tinctoria* ethanol extracts and positive drug gallic acid.

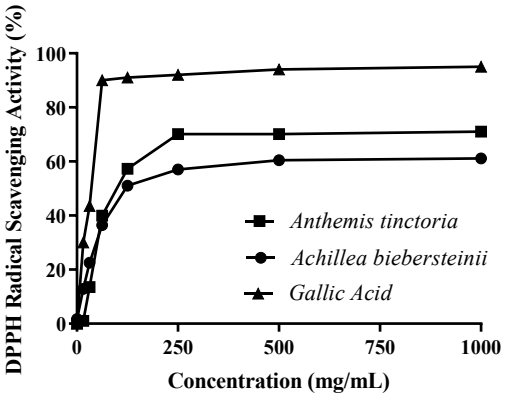
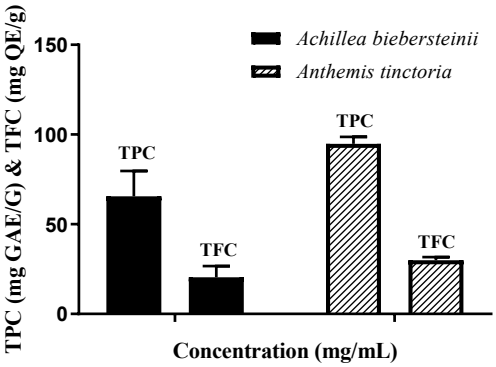


Figure 3. TPC and TFC of *A. biebersteinii* and *A. tinctoria* ethanol extracts.



When the data obtained were evaluated in terms of total phenol and total flavonoid contents, it was determined that both the total phenol and total flavonoid contents of *A.tinctoria* were higher than *A. biebersteinii* (94.9 ± 3.8 mg GAE/g, 65.5 ± 14.2 mg GAE/g for phenol and 29.9 ± 1.8 mg QE/g 20.5±6.3 mg QE/g for flavonoid, respectively) (Figure 3). According to other researchers studying this plant, the total phenol and flavonoid values of methanol extract of *A. tinctoria* L. var. *tinctoria* were determined as 21.4 ± 4.2 (mg GAE/g) and 9.7 ± 2.56 (mg QE/g), respectively (Emir & Emir 2020). Orlando *et al.* (2019) investigated the total phenol and flavonoid components of two species of the genus Anthemis with three different extracts (EA, MeOH, and aqueous) and found that the total phenol and flavonoid content of the MeOH extract of *A. tinctoria* species was higher than that of *A. cretica*. Total phenol and flavonoid ratios in the EA extract of *A. tinctoria* were reported as 26.46 ± 1.11mg GAE/g for phenol, and 45.82 ±

0.40mg GAE/g for the flavonoid. In the current study, conducted with different varieties of the same species, the total phenol content of EA extract was found to be higher. In a previous study investigating the total phenol and flavonoid content of different extracts of *A. biebersteinii* in 2017, it was reported that methanol leaf extract had the highest phenol and flavonoid component (Varasteh-kojourian *et al.*, 2017). In the light of these studies, it can be said that the phenol and flavonoid content of the plants belonging to these species give different results in different parts of the plant, while the solvents in the extracts prepared from the plant can cause a change in the phenol and flavonoid content.

3.3. Enzyme Inhibitory Activity

A. biberstinii and *A. tinctoria* ethanol extracts were tested for the inhibition of AChE and BChE, α-amylase, tyrosinase and α-glucosidase, and the results are given in Table 2. All the enzyme inhibitory activity results are stated as mean ± standard deviation values of three parallel measurements of each group.

Table 2. Enzyme inhibitory activity of 80% ethanol extracts of *A. biebersteinii* and *A. tinctoria.*

Extracts (with 80% ethanol)	Anticholinesterase activity		Antidiabetic activity		Anti-tyrosinase activity
	AChE	BChE	α – glucosidase	α -amylase	tyrosinase
A. biebersteinii	9.51 ± 0.31	24.0 ± 3.42	17.13 ± 1.44	11.29 ± 0.14	70.49±3.34
A. tinctoria	7.88 ± 2.07	8.21 ± 2.03	N.A.	61.58 ± 1.34	72.91±4.69
Referance Drug	58.33 ± 0.44[a]	49.65 ± 0.81[a]	36.48 ± 0.33[b]	53.89 ± 0.15[b]	26.19 ± 0.62[c]

The data are expressed as mean values of three independent experiments ± standard deviation.
a: Galanthamine hydrobromide; b: Acarbose; c: Kojic acid; N.A: Not active

Galanthamine is a reversible inhibitor of cholinesterase and the plant derived drug is used clinically for the palliative treatment of Alzheimer's disease. Therefore, the current study data were compared with galanthamine as a reference drug. The percentage inhibition of AChE was determined to be 9.51 ± 0.31 and 7.88 ± 2.07 for *A.biebersteinii* and *A. tinctoria,* respectively. The percentage inhibition of BChE was 24.0 ± 3.42 and 8.21 ± 2.03, respectively, which was lower than galanthamine (the reference drug) (58.33 ± 0.44 and 49.65 ± 0.81 for AChE and BChE, respectively) at the concentration of 0.1 mg/mL. According to Emir & Emir (2020) methanol extract of *A. tinctoria* L. var. *tinctoria* L. showed 50% inhibition against AChE, BChE and tyrosinase enzymes at the concentration of 254.7 ± 4.86 µg/mL, 166.2 ± 3.74 µg/mL and 415.7 ± 1.85 µg/mL, respectively). In another study, 19.3 and 15.4 mg/ml concentrations demonstrated 50% inhibition on AChE and BChE for *Achillea schischkinii* (Türkan *et al*., 2020). The α-glucosidase inhibitory activity of *Achillea tenorii* has been reported as IC_{50} =32 µg/ml and it has been stated that the strong enzyme inhibitory activity may be attributed to the compound luteolin, which is present in the extract (Venditi *et al.*, 2015). Compared to the previously reported findings, the results of the current study are low, but this may be due to the fact that the solvent and extraction method used will greatly change the amount of phytochemical components contained in the extract.

3.4. Antimicrobial Activity

In the evaluation of the antimicrobial activity of the extracts of the plants used in this study, it was considered significant if MIC ≤ 0.1 mg/ml, moderate if 0.1 < MIC ≤ 0.625 mg/ml, and weak if MIC > 0.625 mg/ml (Kuete, 2010; Awouafack *et al*., 2013).

Table 3. The Antimicrobial Activity Values of 80% Ethanol Extracts of *A. biebersteinii, A. tinctoria.*

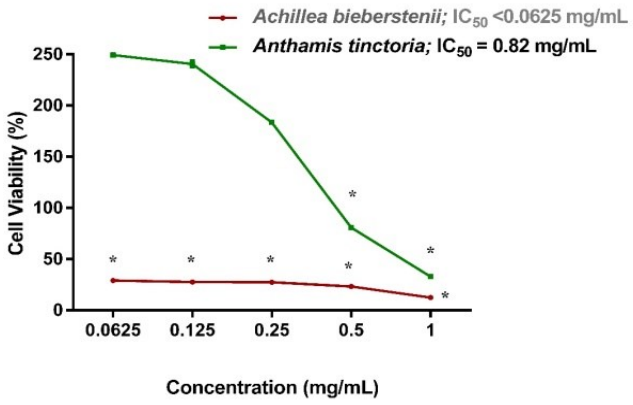
	E.coli	*S.aureus*	*P.aeruginosa*	*B.cereus*	*C.albicans*	*C.tropicalis*
	ATCC 25922	ATCC 29213	ATCC 27853	ATCC11778	ATCC10231	DSM11953
A. biebersteinii	1.25	0.156	1.25	2.5	>2.5	1.25
A. tinctoria	1.25	1.25	2.5	>2.5	>2.5	2.5

According to the results obtained in the study, *A.biebersteinii* extract (0.156 mg/ml) showed a moderate effect on *S.aureus*, while *A. tinctoria* extract did not show antimicrobial activity (Table 3). Kilic *et al*. (2018) used two different methods to evaluate the antimicrobial activity of methanol extracts of *A. tinctoria* and *A. biebersteinii* plants collected from the Amasya region. According to the results of the disc diffusion method, *A. tinctoria* showed higher zone inhibition compared to ceftriaxone antibiotic and generally had antimicrobial properties on *E. coli, P. aeruginosa*, *S. aureus, and K. pneumoniae,* However, in the same study, *A. biebersteinii* species showed antimicrobial activity only on *S. aureus* and *K. pneumoniae*. It was observed that the MIC results, which was the other method applied, provided similar results to the disc diffusion method. Sevindik *et al*. (2018) evaluated the antimicrobial activity of *A. biebersteinii* collected from the Erzincan region. and *A. biebersteinii* was found to have an inhibitory effect on pathogenic microorganisms in the method used, although this effect was lower than that of antibiotics (tetracycline and gentamicin). Uysal *et al*. (2005) reported that *A. tinctoria var. Pallida* did not have significant antimicrobial activity. In the data obtained in the current study, the antimicrobial activity of *A. biebersteinii* supports the findings of studies in the literature, while a difference was determined in respect of the antimicrobial activity of *A. tinctoria* species. The great variability in the results can be attributed to studies having been conducted with different plant extracts and with plants collected from different localities, so there is a clear need for more comprehensive studies.

3.5. Cell Viability Assay

The in vitro cytotoxicity of *A. biebersteinii* and *A. tinctoria* ethanol extracts evaluated using the XTT test are presented in Figure 4. The results from the analysis indicate that *A. tinctoria* extract showed a significant anticancer effect only at its highest concentration (IC_{50}; 0.82 mg/mL) ($p<0.05$). The cell viability results clearly showed that for all concentrations the *A. bieberstenii* extract has a stronger anticancer effect on MDA-MB-231 cells with <0.0625 mg/mL IC_{50} value. Otherwise, the extracts of both plants did not demonstrate significant cytotoxic activity against L929 cells in the applied concentration range (0.0625-1 mg/mL).

Figure 4. The antiproliferative activity of *A. bieberstenii* and *A. tinctoria* extracts.



Similar to the findings obtained in this study, different extracts of *A. bieberstenii* and *A. tinctoria* have been found to have anticancer effects on different cell lines. Baharara *et al.* (2015) reported that silver nanoparticles biosynthesized using *A. bieberstenii* flower extract have an antiapoptotic and inhibitory effect on the MCF-7 cell line. Erdogan *et al*. (2020) reported that the combined form of *A. bieberstenii* with 5-fluorouracil (5-FU) caused inhibition in the human colon cancer cell line.

Studies investigating the cytotoxic effect of *A. tinctoria* are limited. In the research conducted by Raal *et al.* (2022), the methanol extract of this plant was not found to have any cytotoxic effects on the human carcinoma cell lines studied (MKN7, gastric; HepG2, hepatocellular; SW480, colon; KB, mouth; LNCaP, prostate), but the essential oils of the plant had an effective cytotoxic effect on LNCaP and KB cells (IC_{50}: 27.75-29.96 µg/mL). All these data show that the ethanol extract of these two plants provide better results in anticancer studies.

4. DISCUSSION and CONCLUSION

The results of this study demonstrated that 80% ethanol extracts of *A. biebersteinii* and *A. tinctoria* have antioxidant effects (DPPH, ABTS and total phenolic, flavonoid contents), AChE and BChE, tyrosinase inhibitory activity as well as antimicrobial and anticancer activity. Although the antimicrobial activity of *A.bieberstenii* was higher than that of *A.tinctoria*, both showed moderate antimicrobial activity. The antioxidant activity and total phenol-flavonoid content of *A.tinctoria* were found to be higher compared to *A. biebersteinii.* Both extracts showed anticholinesterase inhibitory activity in a concentration-dependent manner. Overall, both extracts showed potent tyrosinase inhibitory activity even from the positive control drug kojic acid at the same concentration. However, more studies are needed to clarify the potential therapeutic effect of *A. biebersteinii* and *A. tinctoria* for tyrosinase-related skin disease or neurodegenerative disease. When the anticancer activities of the plants were evaluated, it was observed that *A. biebersteinii* had a high cytotoxic effect on the breast cancer cell line even at low doses compared to *A. tinctoria*.

The different biological activities of these two plants belonging to the Asteraceae family can be explained by the changes in the phytochemicals in the contents of the plants. In particular, this study has shown that *A. biebersteinii* is a more active in many parameters than *A.tinctoria*. It is expected that the data obtained from these two plants, which have been examined in many ways in terms of biological activity, will shed light on future phytotherapeutic research.

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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

Gulsen Guclu: Investigation, Resources, and Writing - original draft. **Merve Inanir**: Methodology, Supervision, and Validation. **Esra Ucar:** Visualization, Software, Formal Analysis. **Nuraniye Eruygur:** Formal Analysis and Validation. **Mehmet Atas:** Methodology, Supervision, and Validation. **Tansu Uskutoğlu:** Formal Analysis and Validation. **Belgin Coşge Şenkal**: Investigation and Resources

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Research Article

Antioxidant activity, analgesic activity, and phytochemical analysis of *Ammi majus* (L.) extracts

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Abstract: *Ammi majus* (L.) is commonly used to cure many diseases in Moroccan folk medicine, especially vitiligo. This research tries to evaluate the phytochemical constituents of two aqueous extracts (E_1; Maceration; 48 h) and (E_2: Infusion; 1h) and three organic fractions (F_1: Cyclohexane), (F_2: Ethyl acetate (EtOAc)) and (F_3: Ethanolic (EtOH)) of *A. majus* (L.) seeds, as well as to study the antioxidant and analgesic activity of the species. Phytochemical analysis, antioxidant activity (DPPH, FRAP, ABTS, and TAC tests), and analgesic activity (writhing and tail immersion were induced by Acetic acid tests) were analyzed according to the literature. A quantitative phytochemical study indicate that the E_1 had the highest content of total polyphenols (26.95 ± 0.53 mg GAE/g extract) and flavonoids (37.92 ± 0.46 mg QE/g extract), while F_3 showed a promising flavonol content (24.26±0.08 mg QE/g extract). Tannins were found to be high in F_1 (59.27 ± 0.16 mg CE/g extract) and F_2 (57.65 ± 1.18 mg CE/g extract). Antioxidant results reveals that DPPH (IC_{50} = 179.68 ± 0.47 µg/mL) and FRAP (EC_{50} = 367.03 ± 0.12 µg/mL) show to E_1 a high antioxidant activity. Regarding the analgesic activity of the different studied extracts, it was found that E_1 has a high peripheral analgesic effect with 62.32 % and a high central analgesic potential throughout the experimentation at 500 mg/kg. Our studies demonstrated for the first time that *A. majus* seeds extracts have high antioxidant and analgesic activities through different analysis techniques.

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1. INTRODUCTION

Morocco is one of the Mediterranean countries with a long medical tradition and traditional herbal medicine know-how (Bellakhdar, 1997; Bellakhdar *et al*., 1991; Bourhia *et al*., 2019). Due to its unique phytogeographical features, it is considered one of the most important reservoirs of biodiversity for many species. More than 4200 species have been identified,

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including 800 endemic and 600 classified as medicinal plants, which remain largely exploited by the Moroccan population, estimated at between 50 % and 90 % (Bellakhdar, 1997; Fennane & Ejdali, 2016; Merrouni & Elachouri, 2020; Rankou *et al*., 2013; Yamani *et al*., 2015).

Ammi majus (L.) (*A. majus*), commonly called "Atrilal" or "Trillane", "Belala", "Rjel l'aghrabe", and "Ich Omla", is a wild medicinal plant which belongs to the family *Apiaceae* (*Umbelliferae*) (Bellakhdar *et al*., 1991; Rhattas *et al*., 2016; Mohamed *et al*., 2013; Azzouzi, 2015). It is a branched annual plant. It achieves a height of 1.5 - 2.0 meters with whitish-tipped slender roots, and hairless stems with fine longitudinal striae. Generally, the leaves are alternate with a long petiole. The inflorescence is shaded with a small whitish, actinomorphic or zygomorphic, bisexual, pentameric, and bracteate flowers (Humans *et al*., 2007).

In Moroccan traditional medicine, *A. majus* (L.) is widely used to treat several diseases, especially vitiligo, due to its potential dermal effect (Rhattas *et al*., 2016; Redouan *et al*., 2020). Besides, herbalists recommend its use in combination with *Anacylus pyrethrum* to enhance the therapeutic effect on vitiligo (Azzouzi, 2015). Evidence from the literature reveals its curative effect on many cardiovascular diseases (Nassiri *et al*., 2016). The Egyptian population uses the fruit as a diuretic, emmenagogue, and blood purifier, as well as for the treatment of leukoderma, urinary tract infections, and reducing kidney stones (Al-snafi, 2013; El Mofty, 1948; Hakim, 1969; Hawryl *et al*., 2000). In addition, the Iranian population uses *A. majus* against psoriasis and vitiligo, while the Chinese population uses it as a diuretic and carminative, also to treat angina pectoris and asthma (Al-Hadhrami & Hossain, 2016; Asadi-Samani *et al*., 2015).

Indeed, pharmacological research have supported the majority of the plant's traditional uses. According to the literature, the plant has a variety of biological actions, including cytotoxicity (Al-Hadhrami *et al*., 2016; Mohammed & El-Sharkawy, 2017), antibacterial (Al-Hadhrami & Hossain, 2016; Al Akeel *et al*., 2014; Fathallah *et al*., 2019)**,** anti-inflammatory (Koriem *et al*., 2012; Selim & Ouf, 2012)**,** antiviral (Selim & Ouf, 2012)**,** antihyperlipidemic, analgesic, antipyretic (Koriem *et al*., 2012), vascular protector (Cao *et al*., 2020), and antioxidant activities (Al-Hadhrami & Hossain, 2016).

The scientific basis of the pharmacological properties of *A. majus* is based on the diversification of its phytochemical constituents. Several bioactive compounds isolated from *A. majus* are cited in the literature. Some compounds have been isolated from the fruit, including bergapten, imperatorin, xanthotoxin (Bartnik & Mazurek, 2016; M. S. Karawya, 1970), isopimpinellin (Bartnik & Mazurek, 2016), ammajin, marmesin (Balbaa *et al*., 1973), umbelliprenin (Abu-Mustafa EA, EL-Bay FK, 1971b) and maurin (Abu-Mustafa EA, EL-Bay FK, 1971a). Numerous others have been isolated from aerial parts, like 6-hydroxy-7-methoxy-4-methyl coumarin, 6-hydroxy-7-methoxy coumarin (Selim & Ouf, 2012), acetylated flavonol triglycosides, especially, kaempferol and isorhamnetin 3-O- [2''- (4'''- acetylrhamnosyl) – 6''-glucosyl] glucosides, and flavonol glycosides, such as isorhamnetin-3-O-rutferinoside-3-O-glucoside, kaempferinoside-3-O-glucoside, and isorhamnetin-3-O-glucoside (Singab, 1998).

The objective of this work is to assess the antioxidant and analgesic activities of seed extracts of *A. majus* from Morocco. Despite its advantageous pharmacological properties, this species is not well exploited. Currently, the only work that evaluates antioxidant activity uses only the DPPH test, which is still insufficient to prove the antioxidant power of this plant. Its analgesic power is inexperienced. Moreover, its phytochemical composition is not elucidated. It can reveal new compounds with strong antioxidant and analgesic power.

2. MATERIAL and METHODS

2.1. Chemicals

Dimethyl sulfoxide (DMSO), Cyclohexane, Ethyl Acetate, Ethanol, Iron (III) Chloride Hexahydrate, Folin-Ciocalteu, Ascorbic Acid, Gallic Acid, Quercetin, Catechin, Aluminum Chloride, 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and, Acetic Acid, were purchased from Sigma-Aldrich and Solvachim. All other chemicals used were of analytical grade.

2.2. Animals

Winstar Albino rats (160 – 240 g) and *Swiss albino* mice (20 – 30 g) were obtained from the animal house of the Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco. Animals were reared at 22 ± 2 °C with 14 hours of light and 10 hours of darkness, with unrestricted access to food and water.

All experimental procedures were performed in accordance with the "Principles of Laboratory Animal Care" and were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences and approved by the National Institutes of Health (Code of ethics: FMPR0120).

2.3. Plant Materials

The seeds of *A. majus*, had been collected during the fruiting period of May 2019 in Rabat, Morocco (Geographical coordinates: 33° 58' 06" N6° 49' 04" W). Botanical identification of the plant was performed at the Scientific Institute of Rabat. Specimens were stored in the Scientific Institute of Rabat under the herbarium code of RAB111737.

2.4. Preparation of the Extracts

Maceration and infusion

The crushed air-dried seeds of *A. majus* (34 g) were macerated **(E_1)** and infused **(E_2)** in 650 mL of distilled water for 48 h and 1 h, respectively, with intermittent shaking. The extracts were filtered through Whatman filter paper No. 1 (0.45 µm), and then concentrated under vacuum on a rotatory evaporator (BUCHI RE-111 Rotavapor W / 461 Water bath) at 40 °C to constant dryness. The extracts were lyophilized and stored at 4 °C for further analysis.

Soxhlet fractionation

The organic fractions were prepared using a soxhlet apparatus with cyclohexane **(F_1)**, ethyl acetate **(F_2)** and ethanol **(F_3)**, successively for 3 hours for each extraction. At the end of each extraction, the extracts were dried using a rotatory evaporator at 40 °C. Finally, the dried organic fractions were transferred into screw-capped amber vials and stored at 4°C for further analysis.

2.5. Phytochemical Analysis

Total Phenolic Content (TPC)

The determination of total phenolic content was performed using Folin-Ciocalteu method (Poh-Hwa *et al*., 2011). Briefly, we have mixed 200 µL of each extract with 1000 µL of Folin-Ciocalteu reagent at 10 %. Then, we used 800 µL of Sodium Carbonate (Na_2CO_3) at 7.5 % for neutralizing the reaction. The mixture was further incubated in the dark for 30 minutes. The absorbance was determined against a blank at 765 nm with a spectrophotometer (UV-6300PC). A gallic acid standard curve was prepared from a freshly made (From 7 to 125µg/mL) gallic acid stock solution. We have expressed the results as mg of gallic acid equivalents (GAE) /g extract on a dry weight basis (d.w.).

Total Flavonoids Content (TFC-1)

Aluminum chloride colorimetric method was used for the determination of total flavonoids according to the method of (Ordoñez *et al*., 2006). A volume of 0.5 mL of each extract was mixed with 0.5 mL of 2 % Aluminum Chloride ($AlCl_3$) solution. Absorbance was measured 1 h later at a wavelength of 420 nm. Methanol was used as a blank. The calibration curve of Quercetin was obtained in the range of (10 to 60µg/mL). Results were expressed as mg of Quercetin equivalents (QE)/g extract on a dry weight basis (d.w.).

Total Flavonols Content (TFC-2)

The total flavonols content was quantified using the method described by(Yermakov *et al*., 1987). Briefly, 2mL of each extract was mixed with 2 mL of 2 % Aluminum Chloride ($AlCl_3$) solution and 6 mL of 5 % Sodium Acetate solution. Absorbance was measured 2 h 30 min later at a wavelength of 440 nm. Quercetin (15 to 250 µg/mL) was used as standard. The total amount of flavonols was expressed as mg of Quercetin equivalents (QE)/g extract on a dry weight basis (d.w.).

Total Tannins Content (TTC)

The total tannins content (TTC) for each *A. majus* seeds extract was evaluated according to the method described by (Julkunen-Tiitto, 1985). A volume of 50 µL of each extract was added to 1.5 mL of 4 % Methanolic Vanillin solution and 750 µL of concentrated Hydrochloric Acid (37 %). We have incubated the mixture for 20 min in the dark at room temperature. And we have measured the absorbance against a blank (Methanol) at 500 nm. Catechin (100 to 600µg/mL) was used as standard. The tannins content was expressed as mg equivalent of Catechin / g extract (mg CE/g extract).

2.6. Antioxidant Activities

DPPH free radical scavenging activity

To investigate the antioxidant activity of the studied *A. majus* seeds extracts, we used 2,2-diphenyl-1-picrylhydrazil (DPPH) test in similarity with the method established by (Sahin *et al*., 2004). We have mixed 2 mL of Methanolic solution of DPPH at 0.0023 % (60 µM DPPH; Methanol) with 50 µL of each extract at different concentrations. The mixture is vigorously shaken and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting mixture was read at 517 nm. The same procedure was repeated using a control sample (DPPH without extracts). Quercetin (0.38 to 6.09 mg/mL) was used as the standard antioxidant.

The percentage of inhibition was calculated using the formula below. The concentration is plotted as a function of the percentage of inhibition, from the regression equation we calculated IC_{50}.

$$\% \text{ Inhibition of DPPH activity} = \frac{\text{Absorbance of Blank} - \text{Absorbance of Test}}{\text{Absorbance of Blank}} \times 100$$

ABTS free radical scavenging activity

The ABTS radical scavenging assay was performed adhering to the method of (Pukalskas *et al*., 2002). The ABTS radical was generated through the oxidation of ABTS with Potassium Persulfate. In brief, the ABTS solution (7 mM) had reacted with Potassium Persulfate (70 mM) solution (mixed in equal volume) for generation of ABTS cations. The mixture was allowed to stand in the dark at room temperature for 16 h. Before being used in the assay, the ABTS radical cation was diluted with Methanol for an initial absorbance of about 0.700 at 734 nm. For the study, different concentrations of each extract (100 µL) were added to 2 mL of ABTS solution.

The absorbance was read at 734 nm and the percentage inhibition was calculated as described earlier for the DPPH test.

Ferric-reducing antioxidant power test (FRAP)

The reducing activity of the studied extracts was measured spectrophotometrically with the method of (Oyaizu, 1986). We have mixed various concentrations of aqueous extracts organic fractions and standard (0.2 mL), with 2.5 mL of 0.2 M Sodium Phosphate buffer (pH = 6.6) and 2.5 mL of 1 % (w/v) Potassium Ferricyanide ($K_3Fe(CN)_6$). After incubation at 50 °C during 20 min, 2.5 mL of 10 % (w/v) trichloroacetic acid was added to the mixture. About 2.5 mL of each concentration was taken and 2.5 mL of distilled water and 0.5 mL of 0.1 % (w/v) Ferric Chloride ($FeCl_3$) were added. The intensity of the blue-green color was measured at 700 nm. We used Catechin (0.65 to 21.39µg/mL) as a positive control.

Total antioxidant capacity test (TAC)

The total antioxidant activity of aqueous extracts and organic fractions was evaluated by the formation of phosphomolybdenum complex according to the method established by (Prieto *et al*., 1999). 2 mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added to 0.2 mL of each extract. We have measured the absorbance at 695 nm after boiling at 95 °C for 90 minutes. We have choosed Ascorbic acid (15 to 250µg/mL) as a standard. The total antioxidant capacity was expressed as micrograms of ascorbic acid equivalent (AAE) per grams of extract (mg AAE/g extract).

2.7. Analgesic Activity

Acetic acid induced writhing test

The method used in this test has been described by (Koster, 1959). The mice were weighed and randomly divided into 7 groups consisting of 5 mice in each. Group 1 (control) did not receive any treatment. The other groups received orally E_1, E_2, F_1, F_2 and F_3 at 500 mg/kg and aspirin at 125 mg/kg. After 30 minutes of extracts administration, each mouse was injected with 3 % (v/v) Acetic Acid at the dose of 3.75 mL/kg body weight intraperitoneally. The number of abdominal contortions produced by each mouse was recorded for 10 minutes commencing just 10 minutes after acetic acid injection. The percentage of inhibition of abdominal writhing was calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{1 - \text{Number of contortions of mice in the treated batch}}{\text{Number of contortions of the negative control batch}} \times 100$$

Tail immersion test

The tail immersion test was performed according to the method established by (Sewell & Spencer, 1976). Female rats (160 – 240 g) were used in this test. The distribution of the experimental animals remained similar to that of the abdominal writhing test. Briefly, the animals were treated with aqueous extracts **(**E_1; E_2) and organic fractions (F_1; F_2; F_3**)** at 500 mg/kg. Morphine (0.1 mg/kg) was used as a reference standard. The lower 6 cm section of the tail of rats were immersed in a water bath in which the temperature of water was maintained at 55 ± 0.5 °C. The time between tail submergence and tail deflection was recorded at 0, 30, 60 and 120 min after treatment with morphine or extracts using a digital stopwatch. 10 seconds is the time maintained to avoid animal damage.

2.8. Statistical Analysis

Analysis was performed with Graph Pad Prism v8 software. Data shown are the average ± standard deviation of three replicated extractions. Data were subjected to one-way analysis of

variance (ANOVA). Differences between mean values were compared by using Tukey's test at $p \leq 0.05$ probability levels.

3. RESULTS and DISCUSSION

For a rational use of natural resources, the determination of yields has an advantage in deciding the amount of the targeted drug to be taken from nature. The yield results of aqueous extracts and organic fractions of *A. majus* seeds are presented in Table 1.

Table 1. Extraction conditions and yields of obtained extracts from *A. majus* seeds.

Extract Code	Extraction Method	Solvent	Yield (%)
E_1	Maceration (48 h)	Water	17.08 %
E_2	Infusion (1h)	Water	25.47 %
F_1	Soxhlet method	Cyclohexane	8.43 %
F_2	Soxhlet method	Ethyl Acetate	14.44 %
F_3	Soxhlet method	Ethanol	23.35 %

E_1: Aqueous macerated extract; E_2: Aqueous infused extract; F_1: Cyclohexanoic fraction; F_2: Ethyl acetate fraction; F_3: Ethanolic fraction

Regarding the results, the yields decrease with the polarity of the extractive solvents used. Where the higher yield was obtained with the aqueous-infused extract (E_2: 25.47 %), followed by the ethanolic fraction (F_3: 23.35 %), the aqueous-macerated extract (E_1: 17.08 %), the Ethyl acetate fraction (F_2: 14.44 %), and lastly the cyclohexanoic fraction (F_1: 8.43 %). The difference in yields is probably related to the variability of extraction techniques, the time of extraction and the polarity of the used solvents. The comparison of the two aqueous maceration techniques showed that the temperature improves significantly the extractive capacity of water. This could be obviously explained by the fact that the heat causes cell disturbance by increasing the permeability of cell walls, thus enhancing the affinity of water to phytoconstituents. To a certain extent, these results explained the preference of the traditional use of *A. majus* seeds in hot preparations (Bhambri *et al*., 2012; Rhattas *et al*., 2016; Redouan *et al*., 2020).

The objective of the quantitative phytochemical analysis of the studied extracts, using spectrophotometric assays, was to determine their content in total polyphenols, flavonoids, flavonols and tannins. The choice to quantify these phytoconstituents results from its consideration as major contributors to the antioxidant capacity and analgesic potency of medicinal plants. The results obtained are presented in Table 2.

The quantification of total polyphenols revealed that aqueous extracts have significantly high concentrations ($p < 0.05$) in both used extraction methods (E_1: 26.95 ± 0.530 mg GAE/g extract) and (E_2: 23.78 ± 0.420 mg GAE/g extract). In contrast, the organic fractions registered higher tannins content (F_1: 59.27 ± 0.16 mg CE/g extract; F_2: 57.65 ± 1.18 mg CE/g extract; F_3: 16.62 ± 0.28 mg CE/g extract). Significant levels of total flavonoids were observed in aqueous extracts (E_1: 37.92 ± 0.46 mg QE/g extract; E_2: 32.54 ± 0.48 mg QE/g extract), the ethyl acetate fraction (F_2: 33.02 ± 0.29 mg QE/g extract) and the ethanolic fraction (F_3 : 32.36 ± 0.38 mg QE/g extract) with no significant difference ($p < 0.05$). In addition, we found that the ethanolic fraction has a higher concentration of flavonols (F_3: 24.26 ± 0.08 mg QE/g extract) than the aqueous extracts (E_1: 13.91 ± 0.33 mg QE/g extract; E_2: 09.29 ± 0.30 mg QE/g extract), with undetermined value for the cyclohexanoic fraction (F_1) and the ethyl acetate fraction (F_2).

Table 2. Polyphenols, flavonoids, flavonols and condensed tannins contents of extracts from *A. majus* seeds.

Plant Extracts	TPC (mg GAE/g extract)	TFC-1 (mg QE/g extract)	TFC-2 (mg QE/g extract)	TTC (mg CE/g extract)
E_1	26.95 ± 0.53^{a}	37.92±0.46^{e}	13.91±0.33^{g}	7.95±0.02^{j}
E_2	23.78 ± 0.42^{b}	32.54±0.48^{f}	09.29±0.30^{h}	2.27±0.09^{k}
F_1	0.71±0.03c	13.48±0.03^{g}	Nd	59.27±0.16^{l}
F_2	4.74±0.01^{d}	33.02±0.29^{f}	Nd	57.65±1.18^{m}
F_3	4.79±0.01^{d}	32.36±0.38^{f}	24.26±0.08^{i}	16.62±0.28^{n}

Data represent the mean ± standard deviation of three independent experiments.
Values in the same column with different superscript letters indicate significant differences (p-value < 0.05)
E_1: aqueous macerated extract; E_2: aqueous infused extract; F_1: Cyclohexanoic fraction; F_2: Ethyl acetate fraction
F_3: Ethanolic fraction; TPC: Total Phenolic Content; TFC-1: Total Flavonoid Content; TFC-2: Total Flavonol Content
TTC: Total Tannins Content; ND: not determined; mg GAE/g extract: mg Galic Acid equivalent per gram of extract; mg QE/g extract: mg Quercetin equivalent per gram of extract; mg CE/g extract: mg Catechin equivalent per gram of extract.

Comparison with literature showed that the obtained results are in accordance to those found for *Ammi visnaga*, which has the same therapeutic properties as *A. majus* and are cultivated under the same agricultural conditions. In fact, (Muddathir *et al*., 2017) reported a total polyphenols content of 34.1 ± 0.97 µg GAE/g extract in the ethanolic fraction of *Ammi visnaga*. This value is lower than that obtained in our study. For total flavonoids, (Aourabi *et al*., 2021) revealed a lower content in the aqueous extract and organic fractions of the aerial parts of *Ammi visnaga* compared to our study. A number of intrinsic and extrinsic factors, including genetic factors, growth conditions, maturation process, sampling techniques, and storage conditions, probably explain this difference. Indeed, the quantification of secondary metabolites in *A. majus* seeds, such as total polyphenols, flavonoids, flavonols and tannins, permits to identify the potential of its pharmacological properties, including the antioxidant capacity.

Given the complexity of the oxidative processes, the use of different tests is necessary to confirm the antioxidant capacity of the analyzed extracts. For this reason, we proceeded with the evaluation of its antiradical capacity with the DPPH, its reducing power of Iron (FRAP) and Molybdate (TAC), and its antioxidant capacity by ABTS.

The results of the evaluation of the antioxidant activity by DPPH assay (Table 3) indicate that the aqueous extracts exhibit significant DPPH-radical neutralization capacity compared to the organic fractions, with IC_{50} of about (E_1: 179. 68 ± 0.47 µg/mL), (E_2: 198.13 ± 0.28 µg/mL), (F_3: 385.80 ± 0.39 µg/mL), (F_2: 565.04 ± 2.60 µg/mL), and (F_1: 3243 ± 3.65 µg/mL), listed in decreasing order. Based on these values, the antiradical effect of the studied extracts on DPPH-radical is lower than that of Quercetin (5.49 ± 0.02 µg/mL), taken as a positive control. Statistical analyses revealed a significant difference ($p < 0.05$) between the IC_{50} obtained with the five studied extracts and Quercetin.

Similarly, the results issued from other research works revealed lower IC_{50} values of Omanian species, reflecting the strong reducing power of these extracts against DPPH$^{\bullet}$ compared to our extracts (R. Al-Hadhrami & Hossain, 2016). In addition, the comparison with the antioxidant power performed by *Ammi visnaga* via DPPH test, indicates that the organic fractions showed a higher antiradical effect compared to the aqueous extracts, contrary to the expressed capacity of our extracts (Aourabi *et al*., 2021; Bencheraiet *et al*., 2011).

Table 3. Antioxidant activity of the aqueous extracts and organic fractions of *A. majus* seeds and standards using DPPH, ABTS, FRAP and TAC assays.

Plant Extracts	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP EC_{50} (µg/mL)	TAC mg AAE/g of extract
E_1	179.68 ± 0.47 ^{a}	165.07±0.01^{g}	367.03±0.12^{l}	77.25±1.40^{r}
E_2	198.13 ± 0.28 ^{b}	149.93±0.02^{h}	447.31±0.77^{m}	84.68±1.41^{s}
F_1	3243 ±3.65^{c}	3423.8±1.17^{i}	1867.27±1.22^{n}	110.41±2.34^{t}
F_2	565.04 ±2.60^{d}	766.89±9.74^{j}	591.31±4.51^{o}	120.22±1.84^{u}
F_3	385.80 ±0.39^{e}	141.47±0.43^{h}	1128.70±4.58^{p}	120.10±1.13^{u}
Quercetin	5.49 ±0.02^{f}	-	-	-
Ascorbic acid	-	2.52 ±0.02^{k}	-	-
Catechin	-	-	13.90±0.03^{q}	-

Data represent the mean ± standard deviation of three independent experiments.
Values in the same column with different superscript letters indicate significant differences (p-value < 0.05)
E_1: Aqueous macerated extract; E_2: aqueous infused extract; F_1: Cyclohexanoic fraction; F_2: Ethyl acetate fraction
F_3: Ethanolic fraction; IC_{50}: 50% inhibitory concentration of DPPH or ABTS; EC_{50}: Effective concentration that transforms 50 % of Fe^{3+} into Fe^{2+}; mg AAE/g of extract: mg Ascorbic acid equivalent per gram of extract.

The antioxidant capacity against the cationic radical ABTS of the aqueous extracts (E_1; E_2) and the organic fractions (F_1; F_2 ; F_3) was evaluated. Given the IC_{50} values reported in Table 3, the scavenging capacity of ABTS decreases in the following order: Ethanolic fraction (F_3: 141.47 ± 0.43 µg/mL) > Aqueous-infused extract (E_2: 149.93 ± 0.02 µg/mL) > Aqueous-macerated extract (E_1: 165.07 ± 0.01 µg/mL) > Ethyl acetate fraction (F_2: 766.89 ± 9.74 µg/mL) > Cyclohexanoic fraction (F_1: 3423.8 ± 1.17 µg/mL). We conclude that the ethanolic fraction (F_3) and the aqueous-infused extract (E_2) have approximatively the same scavenging capacity of the cationic radical ABTS with no significant difference ($p < 0.05$). Note that the performance of ascorbic acid (2.52 ± 0.02 µg/mL), taken as a positive control, is higher compared to our extracts.

To characterize the reducing power of aqueous extracts (E_1; E_2) and organic fractions (F_1; F_2; F_3) of *A. majus* seeds, two tests were conducted using the Iron (FRAP), Molybdate (TAC) and Reducing Power assessment.

The estimation of the iron reducing power (FRAP) and the total antioxidant capacity (TAC) showed a significant difference ($p < 0.05$) depending on the extraction technique and the solvent used. Based on the results presented in Table 3, the ability of the aqueous extracts (E_1; E_2) to reduce Iron is higher compared to the organic fractions (F_1; F_2; F_3). Thus, this potential decreases in the following order: Aqueous-macerated extract (E_1: 367.03 ± 0.12 µg/mL) > Aqueous-infused extract (E_2: 447.31 ± 0.77 µg/mL) > Ethyl Acetate fraction (F_2: 591.31 ± 4.51 µg/mL) > Ethanolic fraction (F_3: 1128.70 ± 4.58 µg/mL) > Cyclohexanoic fraction (F_1: 1867.27 ± 1.22 µg/mL). These results are consistent with TAC test results, with no significant difference ($p < 0.05$) between the ethanolic fraction F_3 and ethyl acetate fraction F_2.

We found a strong association between the phenolic content measured and the antioxidant ability assessed in the extracts tested, but only with the DPPH radical scavenging test. This finding is explained by the strong scavenging power of radical species and reactive oxygen forms typical to phenolic compounds (Bougandoura & Bendimerad, 2013; Caceres *et al*., 2020). In addition, this capacity depends not only on the polyphenols and flavonoids content, but also on the variability of their structures and interactions in the extracts (Megdiche-ksouri *et al*., 2014).

Another pharmacological activity where the potential of phenolic compounds seems to be interesting is the analgesic activity. Since the search for new analgesics is one of the main therapeutic concerns.

Specific study models were conducted to detect and evaluate the analgesic potential of aqueous extracts (E_1; E_2) and organic fractions (F_1; F_2; F_3) of *A. majus* seeds. The Acetic acid induced writhing test is considered a model of visceral pain, as acetic acid-induced pain is similar to peritonitis (Le Bars *et al*. 2001). This test is frequently used to identify substances with a peripheral analgesic effect. Table 4 summarizes the nociceptive response, indicating the number of cramps performed by the animal after intraperitoneal injection of acetic acid and the percentage of cramp inhibition of the studied extracts.

Table 4. Analgesic effect of aqueous extracts and organic fractions of *A. majus* seeds, and Aspirin on acid-induced writhing in mice.

Treatment	Dose (mg/kg)	Number of writhes	% inhibition
E_1	500	14.00 ± 1.30^{a}	62.32 %
E_2	500	19.50 ± 1.75^{b}	47.52 %
F_1	500	29.00 ± 2.64^{c}	28.13 %
F_2	500	24.50 ± 2.38^{d}	34.06 %
F_3	500	26.00 ± 2.00^{e}	30.03 %
Aspirin	125	21.66 ± 1.90^{b}	41.71 %
Negative Control	-	37.16 ± 1.78^{g}	-

Data represent the mean ± standard deviation of five independent experiments.
Values in the same column with different superscript letters indicate significant differences (p-value < 0.05)
E_1: Aqueous macerated extract; E_2: Aqueous infused extract; F_1: Cyclohexanoic fraction; F_2: Ethyl acetate fraction
F_3: Ethanolic fraction

Regarding the results, the number of acetic acid-induced cramps was significantly ($p < 0.05$) reduced by the aqueous-macerated extract (E_1), administered orally (AO), compared to acetylsalicylic acid (125 mg/kg; AO), taken as a positive control. Besides, at the dose of 500 mg/kg, the antinociceptive activity decreases in the following order: Aqueous-macerated extract (E_1: 62.32%) > Aqueous-infused extract (E_2: 47.52%) > Ethyl Acetate fraction (F_2: 34.06%) > Ethanolic fraction (F_3: 30.03%) > Cyclohexanoic fraction (F_1: 28.13%). Accordingly, we can confirm that the studied extracts of *A. majus* seeds act at the peripheral level. Cytokines, histamine, serotonin and prostaglandins are mediators among several mediators produced by the inflammatory cells under the effect of acetic acid injection, this method is accompanied to the increase of PGE2 and PGF2a in the peritoneal fluid and the production of lioxygenase (Derardt *et al*., 1980). The activity of our extracts may be due to the inhibition of the above mentioned elements. Another study showed that the response induced by acetic acid and depend strongly on both peritoneal macrophages and mast cells (Ribeiro *et al*., 2000).

The tail immersion test was also used to evaluate the analgesic potential of the studied extracts. It is a sensitive model to central analgesics, particularly those with a spinal site of action (Daniel *et al*., 2001). The obtained results, expressed as the mean time of the tail withdrawal reflex at 30, 60 and 120 min, after extracts and morphine treatment at a dose of 500 mg/kg and 0.1 mg/kg, respectively are gathered in Table 5.

Table 5. Analgesic effect of aqueous extracts and organic fractions of *A. majus* seeds, and morphine on nociceptive responses in the tail immersion test.

Treatment	Dose (mg/kg)	Reaction time in seconds			
		0 min	30 min	60 min	120 min
E_1	500	2.43±0.24[a]	5.50±0.79[b]	8.11±0.65[f]	4.39±0.36[l]
E_2	500	2.44±0.10[a]	5.45±0.29[b]	7.39±0.43[g]	5.84±0.79[m]
F_1	500	3.18±0.14[a]	4.82±0.97[d]	4,27±0.79[h]	3.51±0.44[p]
F_2	500	3.21±0.46[a]	3.18±0.51[e]	3.55±0.77[k]	4.24±1.07n
F_3	500	2.88±0.24[a]	4.53±0.61[c]	4.78±0.24[i]	2.5±0.34[p]
Morphine	0.1	2.53±0.43[a]	6.46±0.13[b]	6.75±0.12[j]	7.70±0.18[o]
Negative control	-	2.35±0.32[a]	2.62±0.39[c]	2,63±0.47[k]	2.03±0.31[p]

Data represent the mean ± standard deviation of five independent experiments.
Values in the same column with different superscript letters indicate significant differences (p-value < 0.05)
E_1: Aqueous macerated extract; E_2: Aqueous infused extract; F_1: Cyclohexanic fraction; F_2: Ethyl acetate fraction
F_3: Ethanolic fraction

Based on previous presented results, the oral treatment of rats with the aqueous extracts (E_1; E_2) and organic fractions (F_1; F_2; F_3) significantly ($p < 0.05$) increased the reaction time to the nociceptive thermal stimulus (Fig 2). This antinociceptive effect starts from 30 min and persists throughout the experiment, with a maximal effect at 60 min for the aqueous extracts (E_1: 8.11 ± 0.65 sec) and (E_2: 7.39 ± 0.43 sec) at the dose of 500 mg/kg, comparatively to morphine. The organic fractions showed an antinociceptive effect over the experimental period. The action peaks of cyclohexanoic (F_1: 4.82 ± 0.97 sec), ethanolic (F_3: 4.78 ± 0.24 sec) and ethyl acetate (F_2: 4.24±1.07 sec) fractions were observed at 30, 60, and 120 min, respectively. Antinociceptive activity against temperature-induced pain may be due to stimulation of the opioid receptor or processes facilitated by monoaminergic neurotransmitters such as dopamine, serotonin and norepinephrine that control pain in the dorsal horn (Benarroch, 2008). The intensity of pain can be reduced by activating the downward pain suppression pathway (Dale *et al*., 2005). Several *A. majus* compounds have been identified in the extract of seeds as having central analgesic activity, such as bergapten and, imperatorin (Singh *et al*., 2019). (Koriem *et al*., 2012) found that the ethanolic extract of *A. majus* has a central analgesic activity that confirms what we found.

To a certain extent, this study allowed us to confirm the antioxidant and analgesic properties of the different extracts of *A. majus* seeds, and to scientifically validate the use of this plant in traditional medicine against diseases related to oxidative stress as well as for relieving clinical pain.

The previously presented results indicate that the seeds of this plant contain bioactive compounds with effective antioxidant and analgesic activities *in vitro* that could be used in conventional medicine.

The therapeutic properties of these extracts open a promising way in the fight against oxidative stress and clinical pain. They can constitute a safe and acceptable alternative for human use. For this reason, the evaluation of its toxicity could be considered to assess the risks of their long-term administration. In addition, complementary tests of other pharmacological activities should be conducted to confirm and elucidate the therapeutic performance of this plant.

4. CONCLUSION

The seeds of *A. majus* growing in Morocco could be regarded a source of bioactive chemicals, according to this study. The biological activities of the extracts tested revealed that *A. majus* seeds have a significant anti-oxidant impact and are a prospective source of both peripheral and central analgesics. Given the preceding findings, it appears that A. majus seeds could be used as a source of natural chemicals that could be used in foods, pharmaceutical items, or cosmetics. In perspective to make the best use of the extracts studied, toxicological studies, the chemical composition of each extract and the isolation of certain compounds will be necessary for a future study.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: National Institutes of Health-FMPR0120.

Authorship Contribution Statement

Otman El-Guourram: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Soufiane Drioua:** Investigation, Resources. **Mouna Ameggouz**: Investigation, Resources, Visualization, Methodology. **Najoua Salhi:** Investigation, Methodology. **Karima Sayah:** Resources, Visualization, Software. **Ahmed Zahidi:** Investigation, Supervision, Writing-Reviewing. **Anass Doukkali:** Investigation, Writing-Reviewing. **Gokhan Zengin:** Investigation, Writing-Reviewing. **Hanane Benzeid**: Investigation, Supervision, Writing-Reviewing

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Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Optimization of supercritical fluid CO2 extraction of Opoponax essential oil (C. erythraea)

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Abstract: Essential oil of Opoponax (*Commiphora erythraea*) was extracted by means of steam distillation and supercritical CO_2 (SFE-CO_2) extraction. Experiments were performed using a Clevenger-type apparatus. On the other hand, SFE-CO_2 runs were carried out using OCOLABS extraction unit at the operating conditions of 150, 200, and 250 bars and within temperature range from 50 to 65 °C. Other extraction parameters such as particle size and extraction period were also investigated in order to validate their effect on the processes and the oil yield. The extraction yields were almost similar (2.46%) for the optimized SFE-CO_2 at 250 bar, 65 °C, and 2 hr, and 2.45% for the steam distillation. Our findings confirmed that the grinding of the material (particle size), pressure, and temperature had an important effect on the SFE-CO_2 extraction process. GC/MS analyses revealed that major compounds, namely α-santalene, α and β-bisabolone, and trans-alpha-bergamotene were of similar quantities except for ocimene. α-Santalene concentration was 7.15% for steam distillation and 15.32% for SFE-CO_2 at the abovementioned conditions.

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1. INTRODUCTION

Supercritical fluid extraction (SFE) is an environmental friendly, a fast, and an efficient method for the extraction of many compounds especially in non-polar nature from plant matrices (Tao *et al*., 2014). Carbon dioxide (CO_2) is used under supercritical conditions to extract the principle aromatics from plant materials without using organic solvents. High pressure and temperature transform CO_2 into supercritical carbon dioxide which is neither a gas nor a liquid. In this phase CO_2 supercritical fluid uses both properties being in gas and liquid forms. Therefore, it is able to act as a good and effective solvent. Moreover, after the extraction period, CO_2 quickly and completely evaporates from the extract which is free from solvent residue. It means the process does not require additional steps to remove the solvent itself. Since it is extracted at mild conditions, the resulting oil has an aroma closer to that of the natural plant. As oxidation or degradation during the extraction is mostly prevented, an essential oil mixture obtained from the nature represents the aroma of each oil component and the combination of the aromas gives

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the distinct fragrance. Trace components can be very important. Although their concentration is low in the natural source, they may give the specific scent to the oil. Thus, the natural proportion of the components should be maintained during the extraction of the essential oil from any resources. The supercritical fluid extraction (SFE) technique has remarkable advantages when compared to those of known traditional procedures such as liquid-liquid solvent extraction. Due to the low temperature process, the natural characteristics of the oils and their original components are preserved during the extraction process. As a result of organic solvent free low temperature, extraction and transformation of the components from thermal degradation or hydrolysis can be prevented while solvent contamination is not observed. If controlled extraction conditions are applied, the SFE provides rapid extraction of oils from complex matrices and provides high selectivity for certain compounds (Anitescu *et al*., 1997)**.** There are some important reports in the literature about the selectivity of SFE (Ensieh *et al*., 2007; Khajeh *et al*., 2010; Ouzzar *et al*., 2015).

Opoponax gum resin (*Commiphora erythraea*) is produced in Somalia and known as sweet myrrh and bisabol myrrh because of its earthy-sweet, balsam-like, and lavender-like odour. It is traditionally said to be the noblest of all incense gums. It was used to guard people from negative influences, to strengthen the senses, and to increase awareness and intuition (www.scents-of-earth.com)**.**

The essential oil of *C. erythraea* is used in perfume industry as the class of the Oriental type. The term "opoponax" is confused with other species, *Commiphorae* oil, and with the gum latex oil of some *Umbelliferae*. Most of the published studies related to the analysis of opopanax oil were performed on commercial oils. The first study was reported by Ikeda et al. (1962) on the composition of the essential oil confirming mostly presence of ocimene. Presence of isomeric bisabolenes, and of α-santalene were reported by Nigam and Neville (1968). Following these studies Wenniger and Yates (1969) reported the presence of sesquiterpene hydrocarbons of commercial opopanax oil and along with bisabolenes, they identified other components such as α-cubebene, α-copaene, caryophyllene, β-elemene, *epi*-β-santalene, δ- and γ-cadinene, α- and β-santalene, δ- and γ-elemene, *cis*-, and *trans*-α-bergamotene. Unlike Tucker (1986) some researchers believe that opopanax also belongs to other species such as *C. kataf* (Forsk.) Engl. and *C. guidottii* (Chiov). Therefore, some close morphological similarities of Yemeni shrubs of *C. kataf* to *C. erythraea* are reported (Wood, 1997; Marcotullio *et al*. 2009).

C. erythraea is an industrial plant containing specific essential oil constituents and adds great value to the perfumery industry. Trace components can add distinctive quality to final product if they are present in the preparation. The oil obtained mostly by steam distillation is usually exported to perfume producing companies. The aim of the present work is to compare two different techniques, steam distillation, and supercritical CO_2 extraction of *C. erythraea* essential oil with respect to the extract yields and process time, pressure, and temperature for supercritical CO_2 extraction. The study was also structured to investigate the effects of the particle size of the gum material (ground and unground) on its extraction performance.

2. MATERIAL and METHODS

2.1. Gum Material

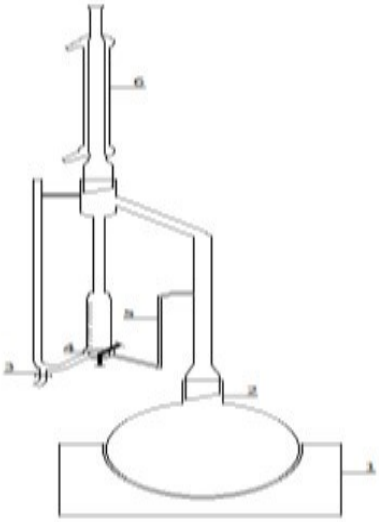
Opoponax gum (*Commiphora erythraea*) was purchased from Neo Gr Limited (England), origin of the product Somalia at Grade 1 and is an amorphous non uniform solid in its natural form (Figure 1). Natural gum is used either directly or ground in a mechanical grinder (15 s) to get a smaller but uniform particle size distribution. 100g of gum is used for the extraction either in the natural form or ground to an approximate powder size (0.2-1mm).

Figure 1. Opoponax (*Commiphora erythraea*) gum in natural form.

2.2. Steam Distillation

The steam distillation experiment was carried out in a Clevenger-type apparatus following the procedure given in the 10th edition of the French Pharmacopeia (Pharmacopée Française). As shown in Figure 2 the system was operated at atmospheric pressure and the mass ratio (1:10) was kept constant by adding water during the process. 100 g of opoponax gum and 1L water were placed in a ball and the mixture was heated to the boiling temperature. The oil was collected over water column concentrated in a flow tube after 4 hours extraction time.

Figure 2. Steam distillation equipment.



1 - Heating mantle, 2 - Two liters flask, 3 - Essential oil output 4 - Three ways valve, 5 - Return tube, 6 – Condenser.

2.3. Supercritical Fluid Extraction (SFE-CO_2) of Opoponax Gum

SFE-CO_2 extraction experiments were carried out at different conditions (temperature, pressure, and time) in the OCOLABS Experimental Supercritical CO_2 Extraction system (USA). The bench scale apparatus consists of CO_2 dip-tube, supercritical fluid extractor; five cylinder separators were assembled in series first with inner volume of 100 mL and the others with 400 mL. The desired pressure and temperature values in the tubes were maintained by feed valve by adequate regulation of CO_2 flow rates, measured by a barometer. When the pressure came to adequate amount in the system, the feed valve was closed.

The filling of the extraction vessel with CO_2 was made directly from a moderately heated cylinder (30-90°C) fitted with a dip tube. 100 g of opoponax was filled to the four cylinder separators for each run. One experiment was also carried out with unground material in order to see the effect of particle size on oil yield.

SFE-CO_2 runs were carried out using OCOLABS extraction unit at the operating conditions of 150, 200, and 250 bars range pressure and at 50-65°C range temperature. Particle size was 0.2-1mm for ground samples and non-uniform 1-2 cm larger particles were also used for extraction. SFE-CO_2 was carried out for 2 or 4 hours at different pressure and temperature to investigate the effect of process periods on oil yield.

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

GC-MS data were obtained using the Agilent Technologies: 7890B GC System Agilent Technologies 5977A MSD equipped with a split-splitless injector (250 ℃) and with a fused silica DB-1 column (60 m; 0:25 mm i.d; 0.25 mm thickness, Agilent J&W). Helium was used as carrier gas, with a rate through the column of 1.0 mL/ min and septum purge of 4 mL/min. The split ratio was 1:100, and the volume of injected samples was 0.1 mL. The GC oven was programmed as follows: first, hold 5 min at 50 ℃ to 220 ℃ with a heating rate 4 ℃/min and then hold 27.5 min at 220 ℃. The interface temperature was 280 ℃. Data acquisition was performed with Enhanced Chem Station for the mass range 40± 400 a.m.u. with a scan speed of 1 scan/sec. The ionization energy of electrons was 70 eV. The identification of compounds was based on a comparison of their mass spectra with NIST (2017) mass spectral library and by relative retention time of compounds identified previously. The percentage composition of the essential oil was computed in each case from GC peak areas without using correction factors.

2.5. AT-FTIR, Color and Refractive Index Analysis

Attenuated total reflectance Fourier transform infrared (ATR-FT-IR) results provided the information about the structural characteristic of the essential oils. ATR-FT-IR spectra of samples were characterized by Agilent Cary 630 ATR- FTIR Spectrometer equipped with the ATR accessory system in 650-4500 cm^{-1} ranges.

Color analysis data were obtained by Konica Minolta CR-5 at room temperature. L, a, and b parameters express the color analysis. The three coordinates were measured by CIELAB in terms of 1) the lightness of the color (L= 0 yields black and L= 100 indicates diffuse white), 2) its position between red and green (a, where negative values indicate green and positive values indicate red), and 3) its position between yellow and blue (b, where negative values indicate blue and positive values indicate yellow). Refractive Index data were obtained by Anton Pear DMS 4500M. Operation condition was at room temperature.

3. RESULTS

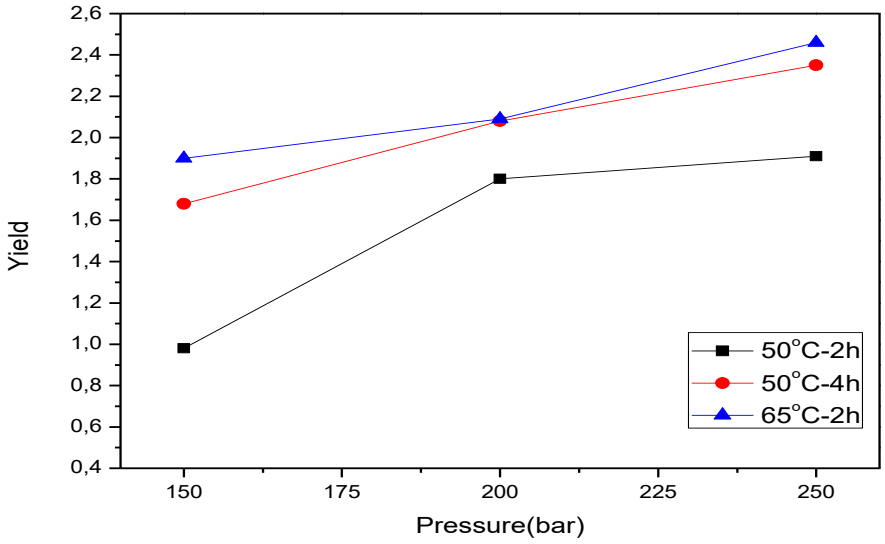
3.1. Extraction Yields: Effect of the Extraction Pressure, Temperature and Extraction Period in SFE-CO_2

Effect of the studied extraction parameters on oil yields are given in Table 1. These results are shown in Figure 3 to make better evaluation. The extraction process is affected from various parameters; therefore, the optimization of the experimental conditions is critical in the development of a SFE method. Generally, applied pressure, temperature, and extraction period are considered as the most important factors during SFE. It is clear from Table 1 that particle size, increased extraction time, and pressure led to a greater amount of the accumulated essential oil. Additionally, the oil yield increased by increasing the temperature at the same extraction period and pressure, which is due to the enhancement of the mass transfer area and provision of shorter diffusion paths.

Table 1. Oil yields (%w/w) of SFE-CO_2 extraction for the parameters studied.

Amount (g)	Pressure (bar)	Temperature (°C)	Time (hr)	Yield (%)	Sample Type
100	150	50	2	0.98	Ground
100	200	50	2	1.80	Ground
100	250	50	2	1.91	Ground
100	150	50	4	1.68	Ground
100	200	50	4	2.08	Ground
100	250	50	4	2.35	Ground
100	150	65	2	1.90	Ground
100	200	65	2	2.09	Ground
100	250	65	2	2.46	Ground
100	250	65	2	0.80	Unground
100				2.45	Steam Distillation (Ground)

Figure 3. Essential oil extraction yield (%) as a function of the pressure, time, and temperature.

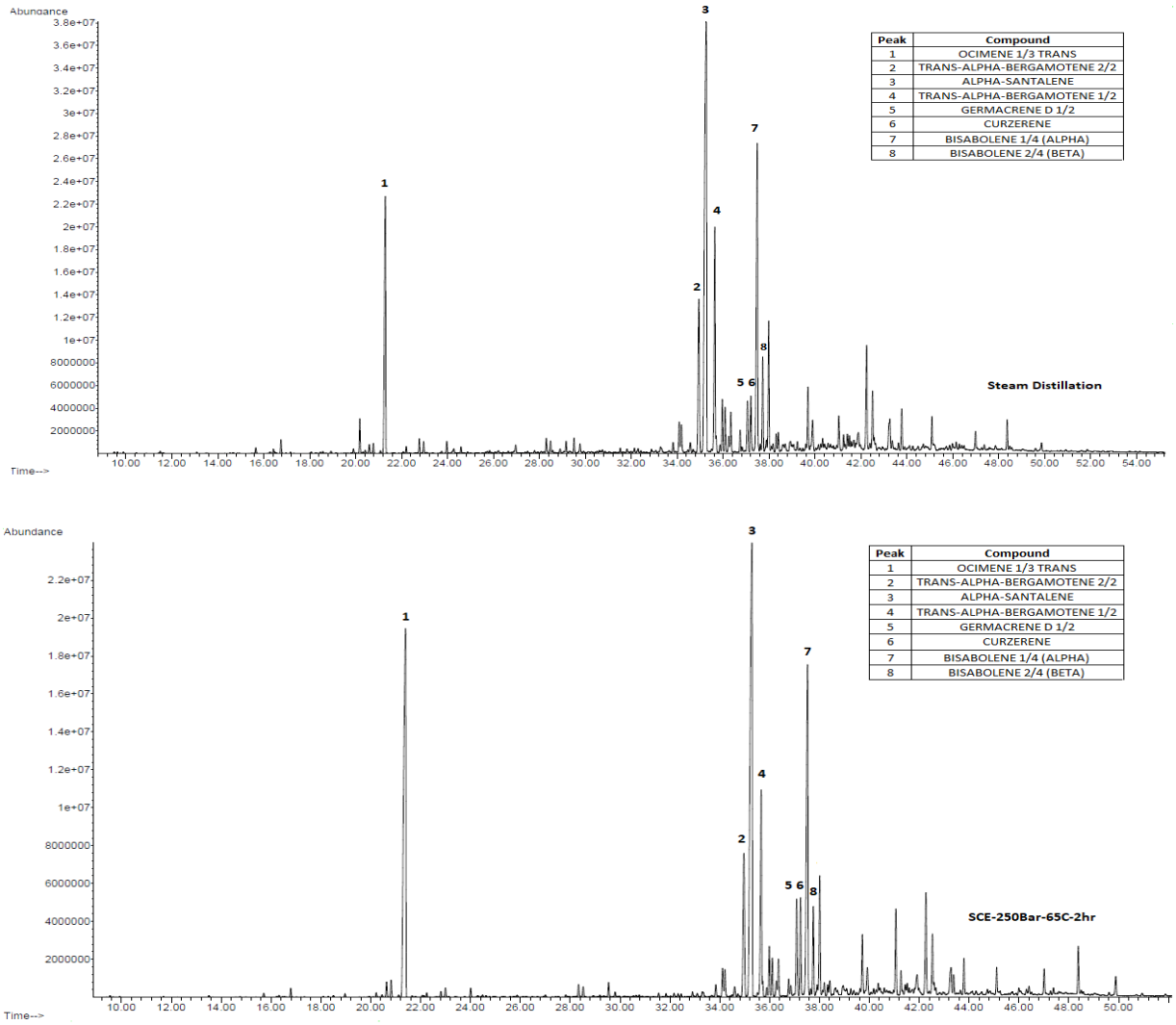


As a result, the decrease of the diameter enhanced mass transfer leading to a better extraction yield. The increased yield of the initial parts of the extraction curves with increasing pressure indicated the accelerated solubility of the oil in supercritical fluid. The yield of opoponax oil increased from 0.98% to 2.46% as pressure increased from 150 to 250 bar. The solubility of the oil in supercritical fluid at high pressure increases the driving force and consequently the mass transfer rate is improved significantly (Özkal, 2004). Similarly, the solubility of the oil in SFE-CO_2 increased by the increasing temperature. The oil yield increased from 1.91% to 2.46% at 250 bar with increase in temperature from 50 to 65 °C after 2 h extraction. Results showed that increasing extraction time to 4 h produced higher extraction yield as recommended in the literature (Anitescu *et al*., 1997). The optimum yield (2.46%) was obtained at 250 bar, 65 °C temperature and 2 hours' extraction period. It should be noted that oil yield was only 0.8% when unground gum is extracted at the same conditions. It shows how grinding has a positive effect providing large surface area that allows the better mass transfer. The oil yield was 2.45% in Clevenger extraction after 4 h steam distillation (Table 1) using the ground gum. This oil was used to make comparisons related to oil components between SFE-CO_2 and steam distillation processes.

3.2. GC-MS Analysis

GC/MS chromatograms of the oils obtained by both methods are given in Figure 4. The 46 compounds were identified and are presented in Table 2. According to GC/MS analyses, similar results were obtained from the steam distillation and supercritical fluid extraction. It is clear that the oil composition was quite similar for major compounds in both of the oil samples; however, some minor compounds were observed in only one process.

Figure 4. GC-MS chromatogram for opoponax oil obtained by steam distillation and SFE-CO_2.



These compounds are α-thujene, sabinene, β-pinene, Δ-careven, carvomenthene, 2-methoxyphenol, cis-verbenol, alcohol C12 dodecylique, furanoeudesma-1,3-diene, and lindestrene for steam distillation and limonene, linalool, methyl acetophenone, 8-hydroxy p-cmene, trans-carveol, α-cubenene, α-longipene, α-copaene, α-humulene, allaoro mandedrene and tau cadinol for SFE-CO_2 extraction. GC-MS analyses revealed that major compounds (α-santalene, α and β-bisabolone, trans-alpha-bergamotene) were of similar quantities except for ocimene. Its concentration was 7.15% for steam distillation and 15.32% for SFE-CO_2, while several important differences were observed between these oils. The percentage of the most desired oxygenated compounds is higher in the oil obtained by SFE-CO_2. Some fatty acids and trace amount of esters were also extracted by this process, but these do not have a negative influence on the oil quality. Organoleptic comparison of the oils showed that the aroma of the oil obtained from SFE-CO_2 was more intense and more pleasant.

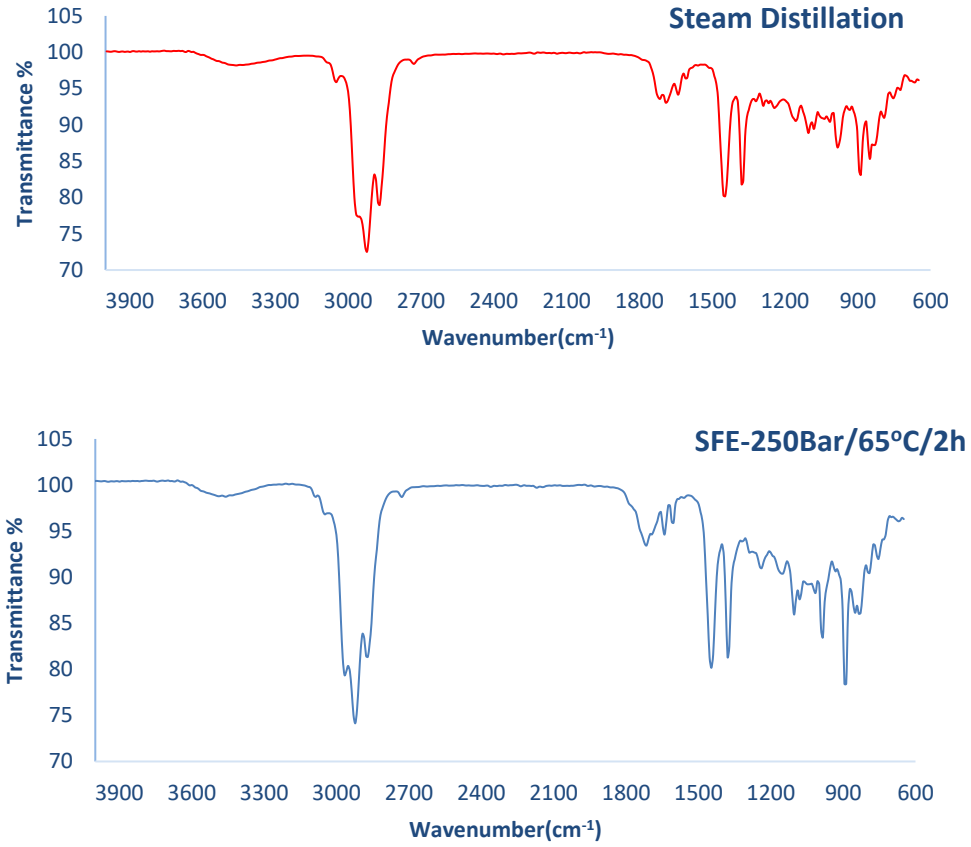
Table 2. Comparative percentages of the identified compounds in opoponax oils.

Compound	Retention Time (min)	Steam Distillation (%)	SFE 250 Bar 65 ºC 2 h (%)	Kovats RI
4,4-DIMETHYL BUTENOLIDE	15.65	0.108	0.075	-
TRICYCLENE	16.28	0.030	0.033	921
ALPHA-THUJENE	16.42	0.080	-	924
ALPHA-PINENE	16.75	0.260	0.164	939
SABINENE	18.25	0.016	-	969
BETA-PINENE	18.46	0.028	-	979
MYRCENE	18.93	0.046	0.062	990
DELTA-CARENE	19.90	0.100	-	1002
PARA-CYMENE	20.18	0.638	0.082	1024
CARVOMENTHENE	20.41	0.064	-	1026
LIMONENE	20.63	-	0.287	-
OCIMENE 2/3 (CIS)	20.77	0.182	0.333	1037
OCIMENE 1/3 (TRANS)	21.29	7.147	15.321	1050
LINALOOL	23.13	-	0.013	-
2-METHOXY PHENOL 1/2	23.77	0.277	-	-
4-ACETYL-1-METHYL CYCLOHEXENE	23.97	0.245	0.172	1130
MYROXIDE 1/2	24.58	0.121	0.031	1132
CIS-VERBENOL	24.88	0.033	-	1141
METHYL ACETOPHENONE 1/3	25.87	-	0.045	1182
8-HYDROXY P-CYMENE	26.13	-	0.015	-
TRANS-CARVEOL	27.52	-	0.020	-
ALCOOL C-10	29.51	0.303	0.276	1269
ALPHA-CUBEBENE	32.91	-	0.117	-
ALPHA-LONGIPINENE	33.1	-	0.077	-
ALPHA-COPAENE	33.84	-	0.268	1376
SESQUITHUJENE (7-EPI) 2/2	34.08	0.695	0.613	1389
BETA-ELEMENE 1/2	34.18	0.617	0.568	1390
SESQUITHUJENE (7-EPI) 1/2	34.57	0.270	0.255	1400
TRANS-ALPHA-BERGAMOTENE 2/2	34.94	4.389	4.047	1412
ALPHA-SANTALENE	35.26	19.167	18.464	1417
TRANS-ALPHA-BERGAMOTENE 1/2	35.63	5.457	4.725	1434
EPI-BETA-SANTALENE	35.96	1.034	0.964	1447
BETA-SESQUIPHELLANDRENE 2/2	36.08	0.891	-	-
ALCOOL C-12 DODECYLIQUE	36.24	0.359	-	1470
ALPHA-HUMULENE	36.27	-	0.350	
BETA-SANTALENE	36.33	0.833	0.756	1459
ALLOAROMADENDRENE	36.54	-	0.039	
AR-CURCUMENE	36.73	0.446	0.336	1480
GERMACRENE D 1/2	37.06	1.388	2.196	1481
CURZERENE	37.20	1.263	2.027	1499
BISABOLENE 1/4 (ALPHA)	37.48	8.588	9.848	1505
BISABOLENE 2/4 (BETA)	37.72	1.932	1.776	1507
DELTA-CADINENE	38.16	0.204	0.294	1523
TAU-CADINOL	40.43	-	0.230	1640
FURANOEUDESMA-1,3-DİENE	41.24	0.900	-	-
LNDESTRENE	41.31	0.448	-	-

3.3. Spectral Analyses

AT-FT-IR spectrums of oils are given in Figure 5. Results show that only minor differences were observed between the extraction methods. Specific absorption bands were identified at: 2929-2914 cm^{-1} and wavenumber region showed C-H band from CH_3 and CH_2, 1647-1640 cm^{-1} (C=C) band/exocyclic methylene groups, 1453-1438 cm^{-1} CH_2 and CH_3 bending bands, 1379-1364 cm^{-1} CH_3 bending bands ,1244-1237 cm^{-1} (C-O) stretching bands,1021-1013 cm^{-1} C-O bending band (Carrión-Prietoa *et al*., 2017).

Figure 5. FT-IR analysis of opoponax oil obtained by both methods.



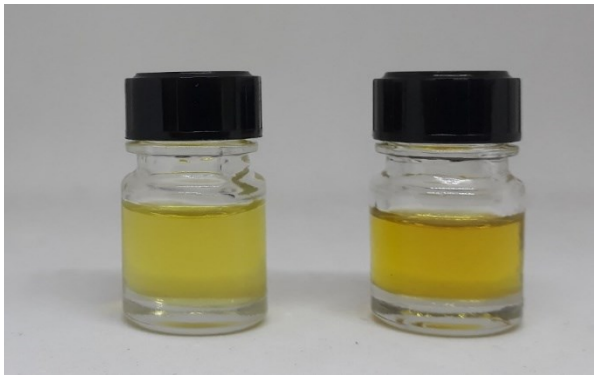
Detailed vibrational frequencies are given in Table 3.

Table 3. Vibrational spectra of opoponax oil.

Frequency (cm^{-1})	Vibration Type
	asymmetric *ν*(CH) from CH_3
2929	asymmetric *ν*(CH) from CH_2
	asymmetric *ν*(CH) from CH_3
1640	ν(C=C) disubstituted olefins; olefinic terpenoids,
	ν(C=C) aromatic ring
1446	*δ*(C-H) from CH_2 or CH_3 groups; CH_2
1379	*δ*$(CH_2)_2$ bending deformation
1238	*δ*(C-H), ν(C-O-H).
	methyl ester, ν(CO), *ν*(C-C)
1021	stretching vibration of C–O ester groups

Color values of the oil obtained from steam distillation are L= 67.82, a=-8.48, b=44.98 and from SFE-CO_2 they are L=87.28, a=-6.4, b=61.98. Apparently, oil color was darker in the latter (Figure 6).

Figure 6. Colors of the essential oils; steam distillation (left), SFE-CO_2 (right).



The refractive index (n) of a material has no universal units but expresses how fast light travels through the material. A higher refractive index corresponds to decreasing the speed of light in the material. The n values are 1.4737 and 1.4896 for steam distillation and SFE-CO_2 opoponax oil, respectively.

4. DISCUSSION and CONCLUSION

The opoponax essential oils were extracted by supercritical fluid extraction and steam distillation method and the results (either as yield% or oil composition) obtained from these two methods have been compared. The SFE-CO_2 was carried out for four parameters, namely temperature, pressure, time, and particle size and the effect of these parameters on the extraction yield was studied. The experiments were designed to obtain a high extraction yield with better organoleptic and spectral properties. The pressure and temperature were the most important factors in the SFE-CO_2. The extraction yields significantly increased as the pressure/temperature increased. The best extraction yield was 2.46% and obtained at 250 bar pressure and 65°C with a mean particle size of 0.2-1 mm. This result was almost the same as the one obtained from the steam distillation. The essential oil obtained from supercritical fluid extraction was deeper in color and odor if compared to the one obtained from steam distillation. The main components of opoponax essential oil were α-santalene and bisabolene 1,4 alpha, however ocimene concentration was two times higher than that in SFE.

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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

Fatih Durak: Investigation, Resources, Experimental, Data Analysis. **Aydin Alemdar:** Investigation, Resources, Experimental, Data Analysis, Writing. **Munevver Sokmen:** Methodology, Supervision, Validation, Experimental, Data Analysis and Writing.

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Research Article

In vitro investigations of biological activities of *Thymus willdenowii* and *Thymus atlanticus* polyphenol-rich extracts

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Abstract: Thyme species produce a wide variety of phenolic compounds including tannins, phenolic acids, and flavonoids. *Thymus atlanticus* (*T. atlanticus*) and *Thymus willdenowii* (*T. willdenowii*) are important thyme species in the southeast of Morocco, with numerous biological properties. The polyphenolic extracts of these two thyme species were obtained using ethanol through Soxhlet apparatus. Antioxidant (DPPH, FRAP, and TAC methods), antihemolytic (2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) induced hemolysis test), hypolipidemic (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity inhibition test), and anti-inflammatory (protein denaturation inhibition) effects of extracts were carried out using *in vitro* methods. The results showed that the polyphenolic extracts of these two species revealed important amounts of phenolic compounds. The contents of flavonoids were significant in the two species, while the contents of tannins and anthocyanin were very low. *T. atlanticus* showed an important antioxidant activity and a considerable antihemolytic effect in AAPH-induced hemolysis test (IC_{50} = 0.29 mg/mL), while *T. willdenowii* showed an important anti-inflammatory activity in heat-induced protein denaturation test (IC_{50} = 1.61 mg/mL). Moreover, both extracts at a dose of 20 µg/mL showed an important *in vitro* hypolipidemic activity by inhibiting HMG-CoA reductase activity (*T. willdenowii*: 51.16 %; *T. atlanticus*: 62.83 %). In conclusion, *T. willdenowii* and *T. atlanticus* extracts have considerable antioxidant, antihemolytic, hypolipidemic, and anti-inflammatory effects. The richness of these species in polyphenols gives them a large specter of biological properties, making them a valuable source of natural bioactive compounds that could prevent or treat various diseases.

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Thymus atlanticus, *Thymus willdenowii*, polyphenols, biological properties.

1. INTRODUCTION

Reactive nitrogen and oxygen species (RNS, ROS) are chemical intermediates with high reactivity, which have built-in chemical properties that confer reactivity to different biological targets (Li *et al.*, 2016; Schieber & Chandel, 2014). The over-production of these chemical elements can oxidize or modify biomolecules, causing several human disorders (Chatterjee, 2016). Antioxidant ability can be defined as the restriction of the oxidation process of bio-molecules such as lipids, deoxyribonucleic acid, proteins, or other bio-molecules. There are two

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types of antioxidants with two different mechanisms. Primary antioxidants have the ability to scavenge the effects of harmful free radicals in a direct manner. In contrast, secondary antioxidants indirectly prevent the generation of free radicals (Atasoy *et al.*, 2019). However, polyphenols can repress the harmful effects of ROS and RNS. These bioactive compounds are puissant antioxidants that provide many health advantages by various mechanisms, including free radical elimination, chelation of pro-oxidant metals, and regeneration and protection of other dietary antioxidant agents (Lima *et al.*, 2014). In addition, polyphenols have attracted more and more attention as powerful antioxidants for treating and preventing the development of pathologies associated with oxidative stress (Li *et al.*, 2014).

Thymus (Lamiaceae family) is considered one of the most heterogeneous genera of the flora of the Mediterranean area (Bartolucci & Domina, 2015). This genus has numerous species and varieties (Rota *et al.*, 2008). It contains more than 336 aromatic species with valuable medicinal properties (Soorni *et al.*, 2019). These species are considered important traditional herbs, widely used in the folk medicine of the Mediterranean area. Several species of thyme are popularly used as condiments, culinary herbs, and ingredients in medicinal products, teas, or syrups, according to their organoleptic, preservative, and medicinal properties (Taghouti *et al.*, 2020). Moreover, thyme herbs are used to treat various pathologies, including upper respiratory infections, coughs, acute and chronic bronchitis, whooping cough, and catarrh (Nabavi *et al.*, 2015).

Thyme plants have several biological effects, including antioxidant, anti-inflammatory, cardio-protective, coagulant, antimicrobial, neuro-protective, anti-carcinogenic, and hypoglycemic properties (El Yaagoubi *et al.*, 2021; Elbouny *et al.*, 2022). The species of this genus are known to possess great quantities of phenolic acids and flavonoids and exhibit potent antioxidant capacity (Ramchoun *et al.*, 2015). Moreover, the phenolic-rich fractions extracted from thyme species are an important source for screening bioactive phytochemicals for possible uses in various areas, including, pharmaceutics, cosmetics, or food industries (Afonso *et al.*, 2020).

In this work, we studied the antioxidant, hemato-protective, anti-inflammatory, and hypolipidemic capacities of polyphenolic extracts of *T. atlanticus* and *T. willdenowii,* to evaluate their bioactivity and to justify their traditional use.

2. MATERIAL and METHODS

2.1. Plant Material and Extraction

Areal parts of *T. atlanticus* and *T. willdenowii* were collected in Errachidia region in April 2021 (32° 15′ N, 5° 25’ E, 1991-2055 m). The species were identified in the National Institute of Agronomic Research of Errachidia. Voucher specimens (Ta HerbFST # 92) and (Tw HerbFST # 93) were deposited in the Herbarium of Faculty of Sciences and Techniques of Errachidia. The plant material was ear-dried in the shade for 7 days before extraction.

Polyphenol extracts were obtained from *T. atlanticus* and *T. willdenowii* aerial parts, as described by Ramchoun *et al.* (2020). Fifty grams of air-dried plant powder was put in a Soxhlet apparatus and defatted with *n*-hexane until the eluent become colorless. Thereafter, *n*-hexane was discarded and the residue was dried. Then, a second Soxhlet extraction was carried out using an ethanolic solution 80% at 45 °C for 4 hours. The obtained hydro-ethanolic extract was dissolved in water and saponins were separated using *n*-butanol. Then, the polyphenolic extract (aqueous fraction) was recovered and concentrated to dryness.

2.2. Determination of Total Polyphenolic Content (TPC)

Polyphenols quantity was estimated using the Folin – Ciocalteu method, described by Singleton and Rossi (1965). A standard phenolic compound (gallic acid) was used to generate the calibration curve (Singleton & Rossi, 1965).

2.3. Determination of Total Flavonoid Content (TFC)

The total flavonoid content of extracts was determined by the aluminum trichloride colorimetric method as described by Khouya *et al*., (2022). To generate the calibration curve, rutin was used a standard flavonoid compound (Khouya *et al.*, 2022).

2.4. Determination of Total Condensed Tannins Content (TTC)

The analysis of condensed tannins was carried out according to the procedure of Heimler *et al.,* (2006). Catechin was used to generate the calibration curve (Heimler *et al*., 2006).

2.5. Determination of Total Anthocyanin Content (TA)

The amounts of anthocyanins in the polyphenol extract was estimated using the pH differential technique (Elisia *et al*., 2007). In brief, 200 microliters of each extract was separately dissolved in KCl (0.025 M, pH 1.0) and sodium acetate (0.4 M, pH 4.5) buffers. Afterwards, the optical densities values of samples were determined at 510 and 700 nm against a blank (water). The absorbance (A) of the sample was determined using the following equation:

$$A = (A_{\lambda vis\text{-}max} - A_{700\ nm})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700\ nm})_{pH\ 4.5}.$$

The amounts of anthocyanins were determined using the following formula:

$$\text{Total anthocyanins (mg/l)} = A \times M \times 1000/(Ma \times C)$$

- M: Molecular weight (449.2 g/mole) of cyanidin-3-glucoside
- Ma: Molar absorptivity (26900)
- C : The concentration of extract in the buffer in mg/mL

2.6. Antioxidant activity

2.6.1. *DPPH radical scavenging activity assay (DPPH)*

DPPH$^{\cdot}$ (1,1-diphenyl-2-picrylhydrazil) radical scavenging antioxidant capacity of extracts was evaluated as described by Elbouny *et al*., (2022). One hundred microliters of each concentration (0,1-1 g/L) of extracts were added to 1 mL of DPPH solution (0.1 mM in methanol). The mixture was incubated for 20 minutes. Then, the optical density was determined at 517 nm versus methanol (Elbouny *et al*., 2022). BHT (butylated hydroxytoluene) was used as an antioxidant reference compound. The following equation was used to determine the percentage of inhibition:

$$I\% = \frac{Ac - At}{Ac} \times 100$$

Where, at is the optical density of test samples, while Ac is that of control.

2.6.2 *Total antioxidant capacity assay (TAC)*

This assay was evaluated using the reduction capacity of molybdenum by extracts at low pH (Prieto *et al*. 1999). Results of TAC assay were represented as mg of ascorbic acid equivalent per gram of extract (AAE mg/gE mean ± STD of three replicates) (Prieto *et al.*, 1999).

2.6.3 *Ferric reducing antioxidant power assay (FRAP)*

This technique was developed to measure the capacity of antioxidants to reduce ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}), based on the method of Benzie and Strain (1999). Ferrous sulfate

(FeSO4, 0.1-1 mM) was used as a reference to generate the calibration curve. Results were represented as Fe^{2+} equivalent (mmol) per gram of extract (Fe^{2+} E mM/gE mean ± STD of three replicates) (Benzie & Strain, 1999).

2.7. Hematoprotective Activity Against (AAPH)-Induced Red Cells Oxidative Hemolysis

The anti-hemolytic effects of thymes was effectuated as described by Kandikattu *et al.,* (2015) with slight modifications (Kandikattu *et al.*, 2015). Blood was collected from male Wistar albino rats in heparin tubes and centrifuged at 4000 rpm for 6 minutes. The lysed erythrocytes were eliminated by repeated phosphate-buffered saline (PBS) wash. Afterwards, 2 % v/v of hematocyte solution was prepared in PBS (pH=7.4). AAPH (2,2'-azo-bis(2-amidinopropane) hydrochloride) which is a peroxyl radicals initiator was used to induce oxidative hemolysis. In clean test tubes, eight hundred microliters of red hematocyte suspension were mixed with 500 µL of extract or ascorbic acid as an antioxidant standard in PBS and 300 µL AAPH (400 mM) were added. Thereafter, the solution was incubated at 37ºC for 3 hours, and centrifuged at 4000 rpm for 6 minutes. Then, the optical density of the supernatant was determined at 540 nm. A control was made by adding 300 µL AAPH and PBS instead of extracts or standard and expressed as 100% hemolysis. The following formula was used to determine the percentage of inhibition:

$$\%\text{Hemolysis inhibition (\%HI)} = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100$$

A_C is the optical density of control and A_S is that of samples.

2.8. *In vitro* Anti-Inflammatory Assay

Thyme extracts' anti-inflammatory effect was effectuated using the method of protein denaturation inhibition as described by Chandra *et al.* (2012) with slight modifications (Chandra *et al.,* 2012). The following solutions were introduced into test tubes: 100 µL of 25% egg albumin in PBS buffer (pH 7), 700 µL of PBS, and 500 µL of varying concentrations of extracts (0.1-1.5 mg/mL). A control was made using distilled water 500 µL. Afterwards, the solutions were incubated (15 minutes at 37°C) and heated (5 minutes at 70°C). After cooling, the optical density at 660 nm was determined. Indomethacin was used as a reference drug in the same conditions as extracts. The following formula was used to determine the percentage of inhibition of protein denaturation:

$$\text{Percentage of inhibition } (\%) = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100$$

A_S is the optical density of test sample and A_C is that of control.

2.9. HMG-CoA Reductase Inhibition Assay

This test was conducted to evaluate the inhibition capacity of the two thymes on HMG-CoA reductase activity. Simvastatin (5 µg/mL) was used as a reference drug. HMG-CoA reductase enzyme assay kit (Sigma-Aldrich) was used.

In an ELISA 96 well plate, 181 µL of 1x assay buffer was mixed with 1 µL of different extracts (20 µg/mL) or simvastatin (5 µg/mL), four µL NADPH solution, 12 µL HMG-CoA substrate, and 2 µL enzyme. Theabsorbance of wells was then measured at 37 °C with a microplate spectrophotometer (Epoch TM 2 microplate spectrophotometer, EPOCH2, BioTek Instruments, USA) at 340 nm wavelength and the absorbance was read every 20 seconds for 10 minutes. For HMG-CoA reductase activity wells, 1µL of 1x assay buffer was added instead of inhibitors. For the blank well, 3 µL of 1x assay buffer was added instead of inhibitors and HMG-CoA reductase solution. The assay was carried out in triplicate for each test well. The activity of HMG-CoA reductase was determined using the following formula as described by the manufacturer.

$$Units/mgP = \frac{(\Delta\text{Absorbance}) \times \text{Tv}}{12.44 \times \text{V} \times 0.6 \times \text{LP}}$$

Where:

ΔAbsorbance: ΔA340/minsample - ΔA340/minblank

Tv: Total volume of the reaction mixture in mL

12.44: Extinction coefficient of NADPH ($2 \times 6.22 mM^{-1}cm^{-1}$) (340 nm).

0.6 = Enzyme concentration in mg-protein/mL (0.6 mg P/mL)

V = Volume of enzyme used (0.002 mL)

LP = Light path in cm (0.55 cm).

2.10. Determination of Inhibitory Concentration 50 Values (IC_{50})

Results were expressed as IC_{50} (50% inhibition) for DPPH, hematoprotective, and anti-inflammatory tests. The concentration that decreases the absorbance of control sample by 50% is defined as IC_{50}. It was determined using the linear regression equation from the inhibition values.

2.11. Statistical Analysis

Data with three groups were analyzed using one-way ANOVA test and those with two groups were analyzed using unpaired t-test. Differences were considered significant when P value is less than (0.05). Results are represented as means± SD.

3. RESULTS

3.1. Determination of Different Polyphenolic Compounds Contents

Table 1 summarizes the findings of the estimation of the total contents of different groups of polyphenolic compounds. The results demonstrated that the polyphenolic fractions of *T.willdenowii* and *T. atlanticus* have an important amounts of phenolic compounds according to Folin Ciocalteu estimation method, *T. atlanticus* had the most significant amounts of these phenolic compounds (p<0.01). Total polyphenols and flavonoids are the most abundant in the extracts of both thymes, whereas tannins and anthocyanins are present in very low quantities.

Table 1. The results of TPC, TFC, TTE, and TA assays .

	TPC (GAE mg/gE)	TFC (RE mg/gE)	TTC (CE µg/gE)	TA (µg/mL)
T. atlanticus	200.91 (±1.78)	76.12 (±0.92)	0.42 (±0.04)	0.36 (±0.09)
T. willdenowii	141.82 (±1.27)	41.7 (±0.78)	0.04 (±0.02)	0.17 (±0.01)
P value	<0.0001	<0.0001	0.0073	0.0024

Values in the same column were analyzed using unpaired t-test. Data are represented as the mean of 3 replicates ± SD.

3.2. Antioxidant Activity

The results of antioxidant assays are represented in Table 2. The two thyme species showed an important antioxidant effect. However, *T. atlanticus* had the strongest antioxidant potential, demonstrated by radical scavenging ability (DPPH), ferric reducing ability (FRAP), and total antioxidant capacity (TAC) assays (DPPH IC_{50} = 0.33 mg/mL, 2.3 Fe^{2+}E mM, and 0.31 AAE mg/gE, respectively) (p<0.01).

Table 2. Result of antioxidant activity.

	DPPH (IC_{50} mg/mL)	FRAP (Fe^{2+}E mM)	TAC (AAE mg/gE)
T. atlanticus	0.33 (±0.01)	2.3 (±0.01)	0.31 (±0.01)
T.willdenowii	0.74 (±0.02)	1.88 (±0.01)	0.28 (±0.01)
Trolox	0.13 (±0.00)	-	-
P value	0.0013	<0.0001	0.0084

Values in the same column were analyzed using unpaired t-test. Data are represented as the mean of 3 replicates ± SD.

3.3. Hematoprotective Activity and Protein Denaturation Inhibition

Both extracts showed that they have an antihemolytic effect against AAPH-induced hemolysis (Table 3). *T. atlanticus* (IC_{50} = 0.29 ±0.01 mg/mL) had a higher hematoprotective effect than that of *T. willdenowii* (IC_{50} = 0.36 ±0.02 mg/ml) which corresponds with the results of antioxidant tests. However, the antihemolytic effect of ascorbic acid (IC_{50} = 0.06 ±0.01 mg/mL) was higher than that of both extracts.

Results of the protein denaturation inhibition assay showed that the extracts of both thymes showed anti-denaturation of protein effect in which *T. willdenowii* had the highest effect (IC_{50} = 1.61 ±0.05 mg/mL) compared to *T. atlanticus* (IC_{50} = 1.91 ±0.04 mg/mL) and indomethacin (IC_{50} = 1.85 ±0.04 mg/mL) (p<0.05).

Table 3. Effect of *Thymus* extracts and standard drugs on protein denaturation and red blood cells hemolysis. Results are represented in IC_{50} value in mg/mL.

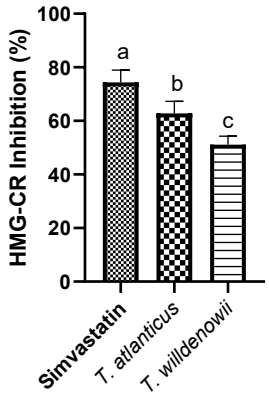
	Inhibition of protein denaturation	Antihemolytic effect
T. atlanticus	1.91 (±0.04)^{a}	0.29 (±0.01)^{a}
T.willdenowii	1.61 (±0.05)^{b}	0.36 (±0.02)^{b}
Indomethacin	1.85 (±0.04)^{a}	-
Ascorbic acid	-	0.06 (±0.01)^{c}

Values in the same column with the same letter group are not significantly different (p<0.05). Data are represented as the mean of 3 replicates ± standard deviation.

3.4. HMG-CoA Reductase Inhibition Assay

The results of HMG-CoA reductase inhibition are shown in Figure 1. All samples have inhibited the activity of the enzyme. However, simvastatin at 5 µg/mL showed the highest effect (74.34 %), whereas *T. atlanticus* exhibited a moderate effect (62.83 %), and *T. willdenowii* had the lowest effect (51.16 %). The three groups had significant different effects (p<0.05).

Figure 1. Values of HMG-CoA reductase inhibition (%) by *T. atlanticus* and *T. willdenowii* extracts or simvastatin. Data are represented as the mean of 3 replicates ± SD. Values of all groups are significantly different (p<0.05).



4. DISCUSSION

Plants synthetize numerous phenolic compounds as secondary metabolites. These bioactive phytochemicals have been known as one of the most widespread groups of plant secondary metabolites with important biological effects related with the prevention of several chronic diseases (Ziaullah & Rupasinghe, 2015). Moreover, several studies have demonstrated that *Thymus* plants are rich in polyphenolic compounds, especially flavonoids and phenolic acids (Boutaoui *et al.*, 2018; Gedikoğlu *et al.,* 2019). Moreover, the plants of this genus showed that they possess a broad variety of active compounds that exert strong antioxidant potential (Bistgani *et al.*, 2019; Labiad *et al.*, 2017; Tohidi *et al.,* 2017). The antioxidant properties of these bioactive compounds may be higher than those of some powerful antioxidant agents such as butylated hydroxytoluene and α-tocopherol antioxidants (Kuete, 2017). However, the antioxidant potential of the extracts of thyme plants can make them a promising source of antioxidant agents.

AAPH is a peroxyl radical initiator that creates free radicals by thermal decomposition. It attacks hematocytes to cause the chain oxidation of lipids and proteins, disturbing the membrane stability which leads eventually to hemolysis (Ramchoun *et al*., 2015). Moreover, AAPH induced hemolysis of red blood cells is an important experimental model for evaluating free radical-induced membrane damage and for measuring the antioxidative properties of several phytochemicals (Takebayashi *et al*., 2007). At the same time, the resistance of red cells to hemolysis exerted by both thyme extracts can be linked to their richness in polyphenols, including flavonoids.

Denaturation of proteins results from alterations in the chemical, biological, and physical characteristics of the protein by disruption of its structure (David Eckersall, 2008). Denaturation of protein leads to the production of autoantigens in conditions such as rheumatic arthritis, diabetes, and cancer which are inflammation conditions. Therefore, inhibition of protein denaturation can inhibit the inflammatory process (Dharmadeva *et al*., 2018). In the present study, *T. atlanticus* and *T. willdenowii* extracts showed anti-inflammatory activity by inhibiting protein denaturation induced by heat treatment. Moreover, several studies have reported that other thyme species exert important anti-inflammatory activities (Lunin & Novoselova, 2010; Shallangwa *et al*., 2016; Ustuner *et al.*, 2019), which makes the plants of this genus an important source of anti-inflammatory compounds.

T. atlanticus and *T. willdenowii* exhibited an important HMG-CoA reductase inhibiting activity. This enzyme is the rate-limiting factor of the mevalonate pathway, responsible for cholesterol biosynthesis. Furthermore, numerous lipid-lowering drugs are used to prevent coronary heart disease by inhibiting HMG-CoA reductase, such as statins. However, these chemical drugs can lead to serious side effects (Sultan *et al.*, 2019). Consequently, using natural extracts to treat and prevent cholesterol-related diseases is a better alternative. Moreover, *Thymus* species possess numerous pharmacological properties, including hypocholesterolemic effects. In a previous study, Ramchoun *et al.,* (2020) reported that *T. atlanticus* lowered cholesterol levels in high-fat diet-fed hamsters. This study showed that the extract of this thyme significantly lowered total cholesterol levels and HMG-CoA reductase gene expression in high-fat-diet fed animals (Ramchoun *et al*., 2020). This study and our study approve that this thyme species can lower cholesterol levels by decreasing the gene expression of HMG-CoA reductase and inhibiting its activity.

The richness of thyme species in phenolic acids and flavonoids can explain their potent biological properties. Flavonoids are important polyphenolic compounds with significant antioxidant properties (Heim *et al.,* 2002). These bioactive compounds have demonstrated in many studies that they have important *in vitro* anti-inflammatory (Rauf *et al*., 2015; Ruiz-Ruiz

et al., 2017; Tasleem & Imam, 2017) and antihemolytic (Naqinezhad *et al*., 2012; Ramchoun *et al*., 2015) activities.

5. CONCLUSION

In this present study, antioxidant, antihemolytic, anti-inflammatory, and hypolipidemic properties of polyphenolic extract of *T. willdenowii* and *T. atlanticus* were carried out using *in vitro* methods. The results showed that these two species have numerous phenolic compounds, including flavonoids. These thyme species had considerable antioxidant, anti-inflammatory, antihemolytic, and hypolipidemic properties. The findings of the present study, along with other previous scientific investigations, confirm that *Thymus* species are an important natural producer of bioactive phenolics with a wide variety of biological properties and applications.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Elbouny Hamza and **Ouahzizi Brahim**: Investigation, resources, visualization, software, formal analysis, and writing -original draft. **Sellam Khalid** and **Alem Chakib**: Supervision, validation, and editing.

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Research Article

Promising antioxidant activity of crude extract from *Calliandra tweedii* Benth

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Abstract: The interest in medicinal plants encourages new research studies on plant extracts, as they are sources for treatments in medicine, agriculture, and veterinary. *Calliandra tweedii* is a native Brazilian species with broad use in folk medicine. This study aimed to examine the leaf crude extract (CE) of C. tweedii and its methanolic (MP) and hexane (HP) phases concerning total phenolic compounds (TPC), proanthocyanidins (PRO), and flavonoid profile, and correlate it to their antioxidant activity (DPPH and FRAP). CE and MP revealed high contents of TPC, PRO and high antioxidant activity (DPPH and FRAP) in relation to HP. Contrarily, HP showed reduced antioxidant activity, according to the scarce phenolic constituents obtained from the partition. Five flavonols were detected by HPLC-UV-DAD, being quercitrin the major constituent. Also, one kaempferol derivate and a series of three quercetin derivates were detected. Strong positive correlations were observed between DPPH, FRAP, and phenolic compounds. These discoveries are important to highlight the promising antioxidant activity of CE of C. tweedii, which could contribute to the expansion of popular herbal medicines and new drug discovery.

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1. INTRODUCTION

Brazil is one of the countries with the largest number of plant species (~50.000), of which half are considered as medicinal. Nevertheless, less than 1% of the plants have been studied concerning their biological activities, so research studies are necessary to confirm their efficacy and reduce the risks of poisoning (Ferreira de Souza et al., 1999). According to the World Health Organization (WHO), 88% of the regions of the Americas recognize the use of traditional medicines due to low cost, affordability, and fewer side effects (WHO, 2019).

Calliandra tweedii Benth, commonly called sponge, belongs to Fabaceae (Mimosoideae) family, genus *Calliandra* (in Greek, beautiful stamens) is one of the 132 species of *Calliandra*,

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ocurrences of shrubs and trees distributed across several countries in South America and Africa (Barbeny & Grimes, 1998). Brazil registers 75 species of *Calliandra*, of which 59 are endemic distributed mainly in the Northeast and Southeast regions (Souza, 2015). *C. tweedii*, earlier called *Inga pulcherrima*, is a Brazilian native species with occurrences in three of the seven phytogeographic domains (Cerrado, Mata Atlântica and Pampa) located in the Southeast (Espírito Santo, Minas Gerais, Rio de Janeiro and São Paulo) and South (Paraná, Rio Grande do Sul and Santa Catarina) regions (Flora do Brasil, 2020). *C. tweedii* is highly sought after by bees and, due to its fast growth, it is used as livestock feed, ornamental plant, garden fences and firewood (Riswan et al., 1996).

C. tweedii has been poorly studied when compared to *C. portoricensis*, *C. calothryrsus,* and *C. haematocephala* (Lorenzi & Souza, 2001; Kumar et al., 2002; Paiva, 2003). In general, people prepare infusions from the bark to use them in problems of rheumatism, arthritis, colds, uterine disorders and edema (fluid retention). In other cases, bark tinctures are made with schnapps for the same purposes (Araujo, 2010).

Previous studies have shown that the active compounds present in plant extracts can mediate biological activities, interacting and modulating the activities of proteins, nucleic acids, and biomembranes. Ethanolic and aqueous extracts of *C. portoricensis* leaves showed constituents identified as tannins, flavonoids, glycosides, and triterpenoid saponins, which inhibited the ulcerogenic effects and gastric lesions in rats at a dose of 50 mg/kg. In addition, these extracts have limited the growth of *Escherichia coli*, *Staphylococcus aureus*, and *S. faecalis* at a concentration of 0.3 to 0.5 mg/mL (Aguwa & Lawal, 1988). Orishadipe et al. (2010) corroborated the phytochemical screening of hexane extract from leaves, stem, and root of *C. portoricensis* reporting the presence of steroids, fatty acids, saponins, and digitalis glycosides with strong antimicrobial activity against *S. aureus*, *E. coli*, and *S. gallinallum*, but not against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. Also, the analgesic activity of the methanolic extracts of the roots and leaves of *C. portoricensis* has been correlated to a dose-dependent of 200, 400, and 600 mg/kg. Da Silva & Parente (2013) reported hemolytic activity from a new triterpenoid saponin of leaves of *C. pulcherrima* and Sikder et al. (2012) described the antimicrobial, cytotoxic, and thrombolytic effect of the methanol extract of *C. surinamensis.*

Additionally, the evaluation of condensed tannins (CT) in *Calliandra* is most important for livestock production, since native species with high CT concentrations (>5% dry matter (DM)) reduce feed intake, digestive efficiency and animal productivity. In contrast, species with moderate concentrations of CT (2-4% DM) improve protein utilization in ruminants (Addisu, 2016). The content and composition of CT can vary according to local weather conditions. CT of *C. haematocephala* and *C. calothyrsus* has been extensively reviewed and correlated to strong antioxidant activity (Rakhmani et al., 2005; Wei et al., 2015).

Considering the lack of studies about the chemical composition of phenolic substances of *C. tweedii* and problems associated with their consumption, this work aimed to investigate the total phenolic compounds, condensed tannins, the profile of flavonoids and their antioxidant activity in crude extract (CE) and methanolic (MP) and hexane (HP) phases of leaves of *C. tweedii.*

2. MATERIAL and METHODS

2.1. Chemical and Reagents

Folin-Ciocalteu reagent, gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH$^{\cdot}$(2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), quercetin, kaempferol, ferric ammonium sulfate, potassium persulfate, ferric chloride, ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate) were purchased from Sigma-Aldrich; sodium carbonate was purchased from

Merck; methanol, hydrochloric acid were obtained from Synth, n-butanol, quebracho tannin. All solvents and chemicals were of analytical or HPLC grade.

2.2. Plant Materials

Leaves of three specimens of C. tweedii were collected around the Institute of Biosciences at the University of São Paulo, São Paulo, Brazil (23°34'1" S, 46°43'49" W; 783 m above mean sea level). A voucher specimen (SPF 185861) was deposited at the Herbarium SPF, Institute of Biosciences, University of São Paulo, SP.

2.3. Preparation of Extracts

Leaves were dried at 40°C for 24 hours and powdered to the extraction process. Dried leaves (30 g) were extracted three times under reflux with 300 mL of 80% methanol for one hour. After pooling the solvent and filtration, extracts were concentrated under reduced pressure at 40°C and then freeze-dried. One hundred milligrams of the freeze-dried extract were taken for analysis as crude extract and the remaining mass was partitioned with 100 mL of methanol and 100 mL of hexane to separate polar and nonpolar substances, respectively. The methanolic and hexane phases were collected and concentrated under reduced pressure at 40°C and immediately freeze-dried. Finally, the crude extract (CE), methanolic phase (MP), and hexane phase (HP) were resuspended in MeOH and submitted to chemical analysis and antioxidant assays.

2.4. Determination of Total Phenolic Compounds

Total phenolic compounds (TPC) were determined using the Folin-Ciocalteu colorimetric method in a microplate reader (SynergyTM H1), according to Furlan et al. (2015). Fifty microliters of CE, MP, and HP solubilized in methanol (0.25 mg/mL) were mixed with 190 µL of ultrapure water, 10 µL of Folin-Ciocalteu reagent, 50 µL of 10% sodium carbonate and incubated in dark for 30 min at 40°C under stirring. The estimation of total phenolic compounds was performed at 760 nm from an analytical curve using gallic acid as a standard (0-80 µg/mL). Results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW).

2.5. Determination of Condensed Tannins

Condensed tannins (CT) content was determined using the HCl/n-butanol method adapted from Waterman & Mole (1994).

In a screw-capped glass tube, 3 mL of 5% HCl/n-butanol was mixed with 100 µL of each sample in the following concentration: CE (2 mg/mL), MP (2 mg/mL) and HP (6 mg/mL) solubilized in methanol. The mixtures were heated at 95ºC for one hour, cooled and the absorbance was read in a spectrophotometer (UV - 1650 PC - Shimadzu) at 550 nm. CT content was estimated using quebracho tannin (0-320 µg/mL) as a standard. Results were expressed in mg of quebracho tannin equivalents (QTE)/ g of dry weight (DW).

2.6. Characterization and Quantification Flavonoids by HPLC

Flavonoids in CE, MP, and HP were investigated using high-performance liquid chromatography (HPLC). Samples (0.5 mg/mL) were solubilized in methanol. CE, MP, and HP were analyzed on an Agilent 1260-HPLC equipped with a diode array detector (G4212B 1260 DAD). Polar substances were separated using a Zorbax C18 column (4.6 x 150 mm, 3.5 µm) (Agilent, Brazil), operated at 40°C. The injection volume for all samples was 3 µL. The mobile phase consisted of 0.1% (v/v) acetic acid in water (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 0-20 min 15% B in A; 20-25 min 15-100% B in A; 25-30 min 100% B in A; 30-32 min 100-15% B in A; 32-35 min 15% B in A. The flow rates were from 0-25 min 1.5 mL/min, 25.1-25.2 min 1.5-1 mL/min, 25.2-26.9 min 1 mL/min, 26.9-27 min 1-1.5 mL/min, 27-35 min 1.5 mL/min. Constituents were monitored at 352 nm and the diode array

detector (DAD) wavelength range was 200-600 nm. Flavonoids were identified by comparison of their retention time with kaempferol, quercetin and quercitrin standards, and UV-Vis spectra.

The amount of each flavonoid was determined in relation to the quercetin calibration curve (3-120 µg/mL). Results were expressed as mg of quercetin equivalents (QE)/ g of dry weight (DW).

2.7. Antioxidant Assays

2.7.1. *DPPH radical scavenging capacity*

Free radical scavenging activity of CE, MP, and HP was determined according to the DPPH radical method by Furlan et al. (2015). DPPH solution in methanol (0.2 mM) was freshly prepared and 200 µL were mixed with 20 µL of each sample at different concentrations (50-250 µg/mL). The reaction mixture was incubated for 20 min at room temperature and in the dark. Absorbance was measured at 515 nm in a microplate reader (SynergyTM H1). As positive controls, Trolox (6-200 µg/mL) and quercetin (7.5-120 µg/mL) were used. Methanol was used as a negative control.

2.7.2. *Ferric reducing antioxidant power* (FRAP)

The ferric reducing power of samples was determined according to Furlan et al. (2015). FRAP solution was prepared daily by mixing 25 mL of acetate buffer (0.3 M, pH 3.6), 2.5 mL of 10 mM TPTZ, and 2.5 mL of 20 mM ferric chloride. Aliquots of 265 µL of FRAP solution were mixed with 10 µL of each sample at different concentrations (50-250 µg/mL) and 25 µL of ultrapure water. After incubation of 30 min at 37°C, the absorbance was detected by a microplate reader (SynergyTM H1) at 595 nm. Trolox (6-200 mg/mL) and quercetin (7.2-180 mg/mL) were used as positive control. Methanol was used as a negative control.

The antioxidant potential of samples was expressed as a percentage of antioxidant activity (%) and the effective concentration of each sample to achieve 50% of the antioxidant activity (EC_{50}).

2.8. Statistical Analysis

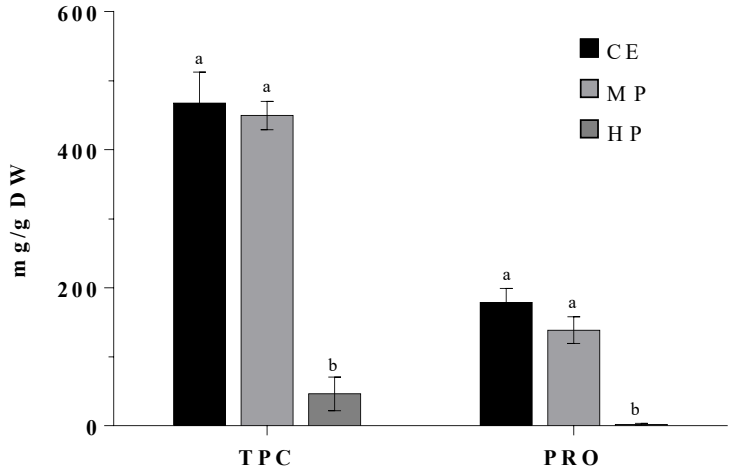
All assays were performed in triplicate and expressed as mean values ± SD (standard deviation). Differences between mean values were assessed by the Tukey's test with a significance level of $p < 0.05$. Data were analyzed using the R Statistics Software (version 3.5.0). Pearson's ($p < 0.05$) was performed for correlation analysis.

3. RESULTS and DISCUSSION

3.1. Total Phenolic Compounds and Proanthocyanidins

Contents of total phenolic compounds (TPC) and condensed tannins (CT) of crude extract (CE), methanolic phase (MP), and hexane phase (HP) of *C. tweedii* are shown in Figure 1. As expected, CE and MP showed high contents of TPC (CE, 467.2 ± 36.5 mg/g DW; MP, 449.2 ± 16.7 mg/g DW; and HP, 46.3 ± 20.0 mg/g DW). Comparing to Firmansyah et al. (2019) and Ahn et al. (1997), who quantified phenolic compounds in *C. calothyrsus* (159.8 mg/g and 188.2 mg/g, respectively), the present results suggest ***C.*** *tweedii* as possessing higher content of phenolic substances than other species from the same genus. However, Wei et al. (2015), evaluating water, 30% acetone, and 70% acetone extracts of *C. haematocephala,* found 199 mg/g, 433 mg/g, and 455 mg/g of TCP, respectively. These last data are very close to those found in this study. Therefore, the content of phenolic compounds could be more related to the extraction methods instead of a defined characteristic for each species.

Figure 1. Contents of total phenolic compounds (TPC, mg GAE/g DW) and total proanthocyanidins (PRO, mg QT/g DW) of crude extract (CE), methanol phase (MP) and hexane phase (HP) of *Calliandra tweedii*. The values correspond to the mean ± standard deviation (n = 3). Different letters represent significant differences among extracts for test.



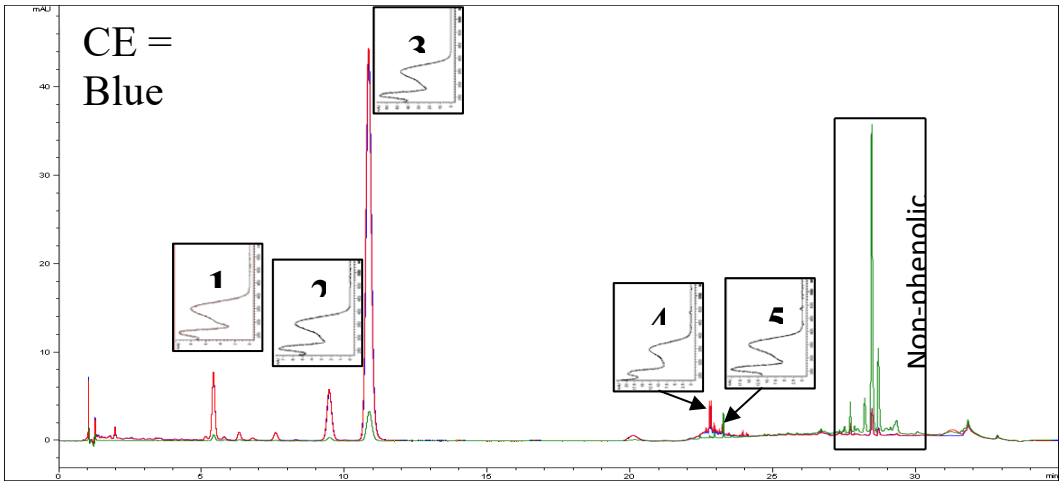
Contents of PRO in CE (178.6 ± 16.7 mg/g DW) and MP (138.5 ± 15.8 mg/g DW) were close to the values reported for *C. calothyrsus* (85.2-117.1 mg/g) (Ahn et al., 1997, Firmansyah et al. 2019), but lower than the ones found in *C. haematocephala* (450-504 mg/g) (Wei et al., 2015). These values suggest that the leaves of ***C.*** *tweedii* could perform a biological activity similar to *C. calothyrsus*, which reduces ruminal methanogenesis due to its high content of tannins (Hess et al., 2004). In addition, these results could be used for discoveries of biological controllers against pathogenic microorganisms that cause great economic losses in horticulture (Hess et al., 2004).

HP phase showed lower content of TPC and PRO, due to the reduced solubility of these compounds in non-polar solvents as hexane. Complementary studies by gas chromatography-mass spectrometry (GC-MS) could be performed with the hexane extract, to test some antimicrobial properties, as Orishadipe et al. (2010) reported for *C. portoricensis*. In any case, partition was effective to eliminate phenolic substances from HP.

3.2. HPLC Analysis of *C. tweedii* Extracts

The profile of flavonoids analyzed by HPLC-UV-DAD from the CE, MP, and HP extracts is shown in Figure 2. The retention times (t_R), the wavelength of maximum absorption (UV λ_{max}), commercial standards, and relevant information about the techniques of flavonoid identification described by Mabry et al. (1970) and Markham (1982) were used to differentiate the flavonoids from leaf extracts of *C. tweedii*. Five flavonols were detected (Table 1).

Figure 2. HPLC-DAD-UV chromatograms at 352 nm of crude extract (CE), methanol (MP) and hexane phases (HP) of *Calliandra tweedii*.



The UV spectrum of flavonol 1 exhibited maximum absorption at 256 and 350 nm and a shoulder at 295 nm, suggesting that it is a kaempferol derivate. Flavonol 2 had UV λ_{max} at 256 and 352 nm and two shoulders at 264 and 308 nm, distinguishing it as a quercetin derivate. Flavonol 3 showed UV λ_{max} at 256 and 348 nm and two shoulders at 264 and 308 nm, identified using co-chromatography with a commercial standard of quercitrin (quercetin 3-*O*-rhamnoside). The UV absorption bands at 256 and 346 nm and two shoulders at 262 and 306 nm of flavonol 4, suggest the presence of a quercetin derivate. Flavonol 5 showed UV λ_{max} at 256 and 350 nm and two shoulders at 264 and 306 nm, reveling another quercetin derivate. This peak 5 was detected in MP and only in one sample of CE and HP (Table 1).

Table 1. Flavonols (mg QE/g DW) detected by HPLC-DAD in *Calliandra tweedii* crude extract (CE), methanol phase (MP), and hexane phase (HP). Values are expressed as means ± standard deviation. t_R: retention time. UV λ_{max} (nm): wavelength of maximum absorption.

Peak	t_R (min)	UV λ_{max} (nm)	Constituent	CE	MP	HP
1	5.35	256, 264sh, 350	Kaempferol derivative	2.5 ± 0.5[a]	2.9 ± 0.6[a]	-
2	9.40	256, 264sh, 308sh, 352	Quercetin derivative	2.0 ± 1.1[a]	2.3 ± 1.1[a]	-
3	10.78	256, 262sh, 308sh, 348	Quercitrin	20.3 ± 6.0[a]	23.6 ± 5.7[a]	0.9 ± 0.9[b]
4	22.44	256, 262sh, 306sh, 346	Quercetin derivative	0.5 ± 0.4[a]	0.6 ± 0.5[a]	-
5	23.25	256, 264sh, 306sh, 350	Quercetin derivative	0.7*	0.3 ± 0.4	0.1*

*Detected in one sample.
For each compound, different letters represent significant differences among extracts.

These data corroborate with the work performed by Moharram et al. (2006), that reported quercetin derivatives as major flavonoids in leaves and stem of *Calliandra* spp., including quercitrin (*C. haematocephala*). These flavonoids have been associated with strong antioxidant potential by scavenging DPPH radical, showing EC_{50} of 18 µM (Yamazaki et al., 2007). Besides that, the antiproliferative and apoptotic activities of quercitrin were demonstrated in colorectal adenocarcinoma cell lines, as well as in lung cancer cells (Cincin et al., 2014). Silva et al. (2012) tested the interaction of the flavonols quercetin, quercitrin, and isoquercitrin with the enzyme arginase from *Leishmania amazonenses* demonstrating that these substances inhibit this enzyme's activity, which is essential for the parasite development. These substances were considered potential molecules for the study of antileishmanial drugs. Furthermore, it has been found the quercitrin efficacy in treating periodontal disease (Gómez-Florit et al., 2014).

Encarnación & Ochoa (1994) isolated and reported antimicrobial activity of two methoxylated flavones (7,2',4',5'-tetramethoxyflavone and 5-Hydroxy-7,2',4',5'-tetramethoxyflavone) from *C. californica.* In a previous study, Encarnación & Keer (1991) observed the antimicrobial activity of ethanol extract of *C. californica* against gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and pathogenic yeasts (*Candida albica*ns).

The estimation of each flavonol amount was summarized in Table 1. Quercitrin (flavonol 3) was the major constituent of CE (20.3 ± 6.0 mg QE/g DW) and MP (23.6 ± 5.7 mg QE/g DW), followed by kaempferol derivative (flavonol 1), quercetin derivate (flavonol 2), and another quercetin derivate (flavonol 4).

3.3. Antioxidant Assays

Antioxidant activity assays of plant extracts have been performed as a form of bioprospecting potential of medicinal plant species, since the prevention of free radical formation, as well as the conversion and stabilization of these molecules in non-oxidizing forms, is essential to prevent oxidative stress. Oxidative stress is associated with many diseases such as cancer, diabetes, atherosclerosis, and autoimmune diseases, among others (Valko et al., 2007).

In this study, the antioxidant activity of *C. tweedii* was evaluated by two different assays: DPPH and FRAP. DPPH is a stable radical, which quench radicals by transferring either a hydrogen atom or a single electron, depending on the antioxidant. The reducing ability of antioxidants toward DPPH radical is measured at 515 nm since the original purple color of DPPH is lost (Prior et al., 2005; Schaich & Xie, 2015). On the other hand, FRAP is a method totally based on the single-electron transference, in which the ferric 2,4,6-tripyridyl-s-triazine (TPTZ) is reduced to an intense blue-colored product (Prior et al., 2005).

The results obtained in the determination of EC_{50} DPPH and EC_{50} FRAP, expressed in µg/mL, are listed in Table 2. In the DPPH assay, EC_{50} of CE and MP were 83.8 µg/mL and 100.5 µg/mL, respectively, while EC_{50} for the standards Trolox and quercetin were 152.2 and 68.4 µg/mL, respectively. In the FRAP assay, EC_{50} of CE and MP were 218.5 µg/mL and 201.6 µg/mL, respectively, while EC_{50} for the standards Trolox and quercetin were 74.8 and 40.6 µg/mL, respectively. EC_{50} of CE and MP were statistically similar; however, these extracts were different from HP in DPPH and FRAP assays.

Table 2. EC_{50} of crude extract (CE), methanol phase (MP), and hexane phase (HP) of *Calliandra tweedii* determined using DPPH and FRAP assay. Values are expressed as means ± standard deviation.

µg/mL	Trolox	Quercetin	CE	MP	HP
EC_{50} DPPH*	152.2	68.4	83.8 ± 9.7^{a}	100.5 ± 1.7^{a}	17259.0 ± 26431.4^{b}
EC_{50} FRAP**	74.8	40.6	218.5 ± 42.5^{a}	201.6 ± 23.6^{a}	3381.2 ± 1103.1^{b}

*EC_{50} was calculated in relation to the negative control; **EC_{50} was calculated in relation to Trolox standard.
For each assay, different letters represent significant differences among extracts.

Considering that CE and MP showed lower EC_{50} compared to Trolox in DPPH assay, and that CE showed an EC_{50} close to the best-known reference antioxidant quercetin (Chen et al., 2013), *C. tweedii* can be considered as a promising source of natural antioxidant compounds. This result highlights the CE extract as promising to carry out complementary biological activities assays.

The results of this study corroborate to those obtained by Adaramoye et al. (2015), who found high levels of inhibition of free radicals by DPPH assay using methanol fraction of leaf extract of *C. portoricensis*. Moharram et al. (2006) associated the observed activity of radical captors in the DPPH test to quercitrin compounds identified in *C. haematocephala*. Therefore,

it is believed that the presence of high levels of phenolic compounds and proanthocyanidins contributed to the high antioxidant activity of CE and MP in this study.

Proanthocyanidins or condensed tannins are polyphenols that play a role in the defense of plants, especially against herbivory (Taiz & Zieger, 2009). Studies evaluating the effects of proanthocyanidins of grape seed extracts have shown that these metabolites have strong antioxidant activity, acting as chemo protectors in rats treated with cisplatin, a potent drug used in the treatment of various types of tumors, but recognized for its toxicity for the patient (Yousef et al., 2009). The effects of proanthocyanidins have also been reported in tests with mice treated with a collagen-induced arthritis compound (Cho et al., 2009). In the same study, animals receiving the plant extract showed decreased inflammation, decreased bone loss and cartilage, and decreased production of H_2O_2, which are parameters of the disease progression.

In many cases, the antioxidant activity is attributed to the synergy among the phenolic compounds in plant extracts. In flavonoids, which have the phenyl rings A and B connected through a pyran C-ring (Dewick, 2009), the antioxidant activity appears to increase with the presence of hydroxyl groups attached to the B-ring. Thus, a single hydroxyl group (OH) substituent can generate little or no antioxidant capacity.

3.4. Correlation Analysis

The analysis of Pearson's correlation coefficient demonstrated how the evaluated phenolic substances of *C. tweedii* might contribute to the antioxidant activity assays DPPH and FRAP (Table 3).

Table 3. Pearson's correlation coefficients for phenolic compounds (TPC), proanthocyanidins (PRO), DPPH, and FRAP with their respective *p-values*. Higher correlation values are highlighted in bold. *P*-values lower than 0.05 were considered significant and appear in italics.

	TPC	PRO	EC_{50}DPPH	EC_{50}FRAP	Flavonol1	Flavonol2	Flavonol3	Flavonol4
PRO	**0.970**							
P-value	***0.000***							
EC_{50}DPPH	-0.562	-0.529						
P-value	*0.115*	*0.143*						
EC_{50}FRAP	**-0.951**	**-0.907**	0.474					
P-value	***0.000***	***0.001***	*0.198*					
Flavonol1	**0.808**	**0.851**	-0.468	**-0.804**				
P-value	***0.008***	***0.004***	*0.204*	***0.009***				
Flavonol2	**0.745**	**0.790**	-0.406	**-0.704**	**0.824**			
P-value	***0.021***	***0.011***	*0.278*	***0.034***	***0.006***			
Flavonol3	**0.890**	**0.895**	-0.507	**-0.831**	**0.880**	**0.959**		
P-value	***0.001***	***0.001***	*0.164*	***0.005***	***0.002***	***0.000***		
Flavonol4	0.486	0.364	-0.298	-0.510	0.418	-0.051	0.202	
P-value	*0.184*	*0.336*	*0.437*	*0.161*	*0.263*	*0.896*	*0.602*	
Flavonol5	0.218	0.142	-0.142	-0.246	0.402	0.509	0.470	0.114
P-value	*0.574*	*0.715*	*0.715*	*0.523*	*0.284*	*0.162*	*0.202*	*0.771*

EC_{50} of FRAP assay showed higher negative correlations for TPC and PRO (-0.951 and -0.970, respectively, *p-value* $\leqslant$0.001), compared to flavonols 1, 2 and 3 (-0.704$\leqslant$r$\leqslant$-0.831, *p-value* $\leqslant$0.034). This result could indicate that phenolic compounds other than flavonoids influence more the antioxidant activity. Among these phenolic compounds, we can point out the PRO content, which presented higher negative correlation with EC50 of FRAP assay than any other flavonoid. Since we have focused on the analysis of individual flavonoids instead of

proanthocyanidins, a deeper study concerning the later substances could confirm this promising antioxidant activity from leaves of *C. tweedii*. Flavonols 4 and 5 showed no significant correlation with TPC, EC_{50} DPPH, EC_{50} FRAP and flavonols 1, 2 and 3 ($-0.051 \leqslant r \leqslant 0.509$, *p-value* >0.16), probably due to its reduced content in CE, MP, and HP. Phenolic compounds are recognized as potent antioxidants and have already demonstrated moderate to strong scavenging properties on DPPH assay by leaves of another *Calliandra* species. More specifically, the antioxidant activity has been correlated with quercetin rhamnosides detected in these species (Moharram et al., 2006). Although we have identified quercetin derivatives in the methanol extract and phase, the correlation analysis pointed out that the proanthocyanidins could be responsible for this role.

Also, no significant correlation between FRAP and DPPH was observed (r=0.474, *p-value* = 0.198). This is an intriguing result since the basic principle of both antioxidant assays is to measure the capacity of the extract constituents in transferring an electron and in reducing an oxidant compound.

4. CONCLUSION

To our knowledge, *C. tweedii* flavonoids have not been reported in other studies, being this work precursor in providing important data regarding preliminary identification to further elucidate the molecular structure of these flavonoids. It is important to highlight that the antioxidant activity of the crude extract (CE) and the methanolic phase (MP) are promisors and they were not statistically different from each other. In addition, their EC_{50} values were similar to quercetin's. Therefore, a partition of the crude extract would not be mandatory in order to obtain a potent antioxidant extract. This is an interesting aspect because higher amounts of material can be wasted in the partition process. These results reveal a potential use of leaves of *C. tweedii* as herbal medicine. Although flavonoids, mainly quercetin rhamnosides, have already been related to the promising antioxidant activity, the correlation of the antioxidant assays from this study was higher with TPC and PRO. Therefore, more studies must be performed to investigate the role of other phenolic compounds in the antioxidant activity of *C. tweedii*.

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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

This study was developed as a part of the III Winter Botany course offered by the Botany Department of the University of São Paulo. **Kátia Dos Santos**: Conceptualization, Investigation, Formal analysis, Writing – original draft; **Alice Nagai**: Investigation, Formal analysis, Writing – original draft, Writing – review & editing; **Carmen Palacios**: Investigation, Formal analysis, Writing – original draft, Writing – review & editing; **Bruno Evangelista**: Formal analysis, Writing – original draft; **Carlos Priante**: Formal analysis, Writing – original draft; **Débora Zamban**: Formal analysis, Writing – original draft; **Claudia Furlan**: Writing – review & editing, Supervision.

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Research Article

Analysis of phytochemical composition and biological activities of *Helichrysum pallasii* (Sprengel) ledeb leaves

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Abstract: This study aimed at the investigation of *in vitro* biological activities (antioxidant and enzyme inhibition) and phytochemical composition of various extracts (ethanol maceration, aqueous infusion, and aqueous decoction) obtained from leaf organs of *Helichrysum pallasii*, which has been traditionally used as herbal tea and medicine in Eastern Anatolia.

Experimental analysis showed that ethanol-based extract had higher total phenolic content (TPC: 103 mg GAE/gE) and showed superior antioxidant potentials (FRAP: 2205 µmol Fe2+/gE; ORAC: 2540 µmol Trolox Eq./gE; DPPH: IC50=0.58 mg/ml; CUPRAC: IC50=0.37 mg/ml; Phosphomolybdenum: IC50=1.34 mg/ml ve metal chelation: IC50=1.42 mg/ml) and enzyme inhibition (Acetylcholinesterase: IC50=1.49 mg/ml; Butyrylcholinesterase: IC50=1.98 mg/ml; Tyrosinase: IC50=0.68 mg/ml; Alpha-amylase: IC50=2.09 mg/ml; Alpha-glucosidase: IC50=0.51 mg/ml; and Pancreatic lipase: IC50=42.5 µg/ml) and contained higher amounts of phenolic (chlorogenic acid isomers and rutin) and fatty acid (palmitic, linoleic, and linolenic acids) compounds than traditional preparations (infusion and decoction).

The current study's findings indicate that the leaves of the *Helichrysum pallasii* are a source of phytochemicals with strong antioxidant and enzyme inhibitory properties, implying that it could be a candidate for biotherapeutic agent research and development.

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1. INTRODUCTION

The ethnobotanical knowledge in the Anatolian area consists of the blending of the ethnobotanical knowledge of the native and immigrant peoples due to the rich vegetation, the high rate of endemic plants, and the migration of various tribes and nations from other lands to Anatolia, along with their knowledge and usage patterns. Especially the Eastern Anatolia

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Region is home to a vibrant culture in terms of ethnobotany (Altundag and Ozturk, 2011; Dalar *et al.*, 2018).

This study chose *Helichrysum pallasii* (Asteraceae) (Figure 1), which has been used in traditional medical treatment for a long time in the Eastern Anatolia Region. In Eastern Anatolia, *Helichrysum pallasii* is called as Ölmez çiçek, Altın çiçek, Altın otu, Herdemtaze, and Herdemcan. It is commonly used as folk medicine to treat diabetes, epilepsy, colds, and respiratory disorders. Taxa of *the Helichrysum* genus in Anatolia are used internally, primarily by preparing herbal tea by infusion or decoction (Baytop, 1999; Mükemre *et al.*, 2015; Dalar *et al*., 2018, Dalar and Mükemre, 2020).

Herbal teas have been widely used in the daily lives of various cultures for their health-promoting activities. They are among the primary sources of dietary antioxidants in the diet of many cultures due to the presence of phenolics, vitamins, and carotenoids (Piljac-Žegarac *et al*., 2013). Herbal tea process is among the oldest and simplest forms of drug administration known in herbal therapy. The plants used as herbal tea are collected during the vegetation period, dried in the dark and stored for later use (Altundag and Ozturk, 2011; Dalar *et al.*, 2018; Dalar and Mükemre, 2020). Bioactive components can interact more effectively with organic solvents. These solvents are thought to deform the cell wall and vacuole membrane more effectively, enabling the release of compounds in the plant sap. Dai and Mumper (2010) propounded that ethanol solvent is highly effective in extracting plant bioactive components.

Despite extensive utilization as infusion and decoction, there is only limited data regarding the chemical composition and biological activities of organic solvent-based extracts obtained from *the Helichrysum* genus in the literature (Carini *et al*., 2001; Süzgeç *et al*., 2005; Albayrak *et al*., 2008; Kolaylı *et al.*, 2010; Albayrak *et al*., 2010; Jahromi *et al*., 2017). Therefore, this study aimed to (i) identify phytochemical composition by using HPLC-MS/MS and GC-MS and (ii) measure total phenolic levels and antioxidant capacities by using complementary methods (FRAP, ORAC, DPPH, Metal chelation, CUPRAC, and Phosphomolybdenum) of traditional preparation methods (infusion and decoction) and ethanol-based extracts obtained from *Helichrysum pallasii* leaves comparatively.

2. MATERIAL and METHODS

2.1. Plant materials

Leaf samples of *Helichrysum pallasii* (Figure 1a) were collected from the rocky slope, steppe habitats in the hamlet of Batkan (Tanriverdi), the village of Konalga, Çatak, Van, Türkiye on June 24th, 2019 (Global Positioning System (GPS) coordinates 37° 50' 080" N, 43° 12' 412" E, 2163 m) and transferred to the laboratory. Botanical identification of the plant samples was carried out at Van Pharmaceutical Herbarium (VPH), Faculty of Pharmacy, Van Yuzuncu Yil University, Turkey, and the voucher specimens were stored at VPH (Herbarium code: VPH-548, Collector Code: DM-345) (Figure 1b). Plant materials were divided, adequately cleaned of dust and contaminants, and left at room temperature in the dark until dry (Figure 1c). Subsequently, the samples were ground into a fine powder using a grinding mill (Isolab laboratuar mill 602, Interlab, İstanbul, Turkey) and stored at -20°C for a maximum of 4 weeks until analysis.

Figure 1. *Helichrysum pallasii* (Sprengel) Ledeb. **a.** General view of the plant; **b.** Herbarium specimen; **c.** Dried leaf samples.

a	b	c

2.2. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA) and were of analytical or HPLC grade.

2.3. Preparation of Extracts

2.3.1. *Ethanol extract*

The ethanol-based lyophilized extract was prepared as described previously (Dalar and Konczak, 2013). Briefly, the ground plant material was mixed with a 10-fold volume of acidified ethanol (80% ethanol and 1% HCl (v/v) in water) shaken for 2 h at room temperature (22°C), and centrifuged at 15,320 x g (10000 rpm in a Beckman JA14 (137 mm) rotor, Sorvall RC-5B centrifuge, Wilmington, DE, USA) at 4°C for 20 min, after which the supernatant was collected. Subsequently, the supernatant was individually evaporated using reduced pressure at 37°C and a rotary evaporator (Rotavapor R-205, Buchi, Flawil, Switzerland). The concentrated fraction was freeze-dried using a lyophilizator (Alpha 1-2 LDplus, Christ, Osterode am Harz, Germany) under a vacuum at -51°C to fine lyophilized powder stored at -20°C for a maximum of 4 weeks until being analyzed.

2.3.2. *Herbal infusion extract*

The herbal infusion was prepared according to Baytop (1999). Briefly, plant materials (leaf) were mixed with a 10-fold volume (g/ml) of preboiled distilled water and incubated for 10 min. The mixture was filtered using cotton and vacuum filtering (45 µm). The filtrates were evaporated and freeze-dried as previously described.

2.3.3. *Herbal decoction extract*

The decoction was prepared according to Baytop (1999). Briefly, the leaf powder was mixed with a 10-fold volume (g/ml) of cold distilled water and heated to boiling. The mixture simmered for 3 min, then was placed aside to cool over the next 10 min and processed like the herbal infusion.

2.4. Total Phenolic Content

The extracts' phenolic content was measured using the Folin-Ciocalteu method, as described previously by Ainsworth and Gillespie (2007). It was expressed as mg gallic acid equivalents per gram of dry weight of the lyophilized extracts, based on the gallic acid standard curve and against a blank control. The analyses were conducted in triplicate.

2.5. Antioxidant Capacity

Ferric reducing antioxidant power (namely total reducing; FRAP) assay was conducted according to Benzie and Strain (1996), and total reducing capacities of the extracts were expressed as µmol of iron (Fe^{2+}) per gram of dry weight of lyophilized extracts based on an iron sulphate standard (Fe_2SO_4) curve against a blank control. The extracts' oxygen radical scavenging (ORAC) capacities were measured as described previously by Dalar & Konczak (2013). Based on a Trolox standard curve, the results were expressed as µmol Trolox equivalent per gram of dry weight of the lyophilized extract.

The total antioxidant (TAC; phosphomolybdenum method), DPPH radical scavenging, ABTS radical cation scavenging, cupric ion reducing (CUPRAC), and metal chelating activities of the extracts were determined as previously described by Uysal *et al*. (2017) and the results were expressed as IC_{50}-half maximal inhibitory concentration (mg extract/ml).

2.6. Enzyme Inhibitory Activities

Cholinesterase (ChE), α-amylase, α-glucosidase, and tyrosinase inhibitory activities of the extracts were determined according to Zengin (2016) and expressed as IC_{50}-half maximal inhibitory concentration (mg extract/ml). The pancreatic lipase activity was assayed as described previously (Dalar and Konczak, 2013) using 4-methylumbelliferyl oleate (0.1 mmol) as a substrate, and the results were expressed as IC_{50}-half maximal inhibitory concentration (mg extract/ml). All inhibitory enzyme analyses were conducted in triplicate.

2.7. Chemical Composition

2.7.1. *HPLC-MS/MS analysis*

Identification and quantification of phenolic compounds by high-performance liquid chromatography-diode array detector (HPLC-DAD) and liquid chromatography-photo-diode array–mass spectrometry (LC-PDA–MS/MS) (Thermo Fisher Scientific, Waltham, MA, USA) analysis were conducted as described previously (Dalar and Konczak, 2013). The composition of phenolic compounds was characterized based on their UV spectrum, retention time, co-chromatography with commercial standards, when available, and MS/MS fragmentation patterns. MS experiments in the full scan (parent and product-specific) and the selected reaction monitoring (SRM) mode were conducted. All analyses were conducted in triplicate.

2.7.2. *GC-MS Analysis*

Volatile compounds and fatty acids were analyzed by gas chromatography-mass spectrometry (GC/MS) (3800 Varian GC, Agilent Technologies) using a headspace solid-phase microextraction and identified by the fragment ions and relative retention indices of their peaks with those of the MS library standards as described previously (Uzun *et al*., 2017). All analyses were conducted in triplicate.

2.8. Data Analysis

The mean values were calculated based on at least three determinations (n=3). One-way ANOVA followed by the Bonferroni *posthoc* test was performed to assess differences between the samples at $p<0.05$ through Graphpad Prism 5 (Graphpad Software, CA, USA).

3. RESULTS and DISCUSSION

3.1. Phytochemical Composition

The highest extraction yield was determined in infusion (24.4%) and decoction (26.2%) extracts, and the lowest yield in ethanol extract (16.2%); however, when the results of antioxidant and enzyme inhibition activities were examined, it was observed that the highest values were in ethanol extracts. The phytochemical composition of the extracts was analyzed

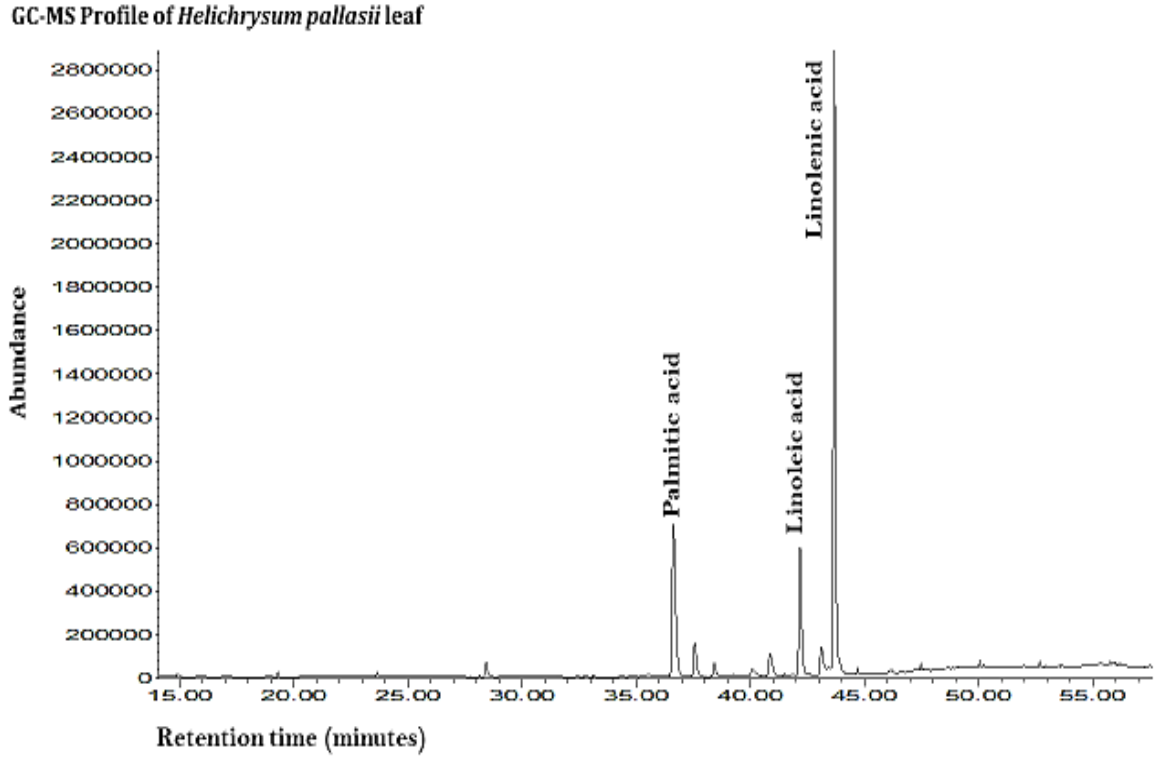
to detect significant contributors to antioxidant and enzyme inhibition activities. The fatty acid compositions of the extracts were determined using GC-MS, and the phenolic compound compositions were determined using HPLC-MS/MS. As presented in Table 1 and Figure 2, the fatty acid compositions of the extracts consist of three main components, namely palmitic, linoleic, and linolenic acids in the ethanol extract; and two fatty acid compounds, palmitic and linolenic acids were detected in infusion and decoction extracts. Palmitic and linolenic acids were determined as the most dominant fatty acids in the extracts. These findings are consistent with previous chromatographic results of *Helichrysum* species (Albayrak *et al*., 2008). This finding showed that *Helichrysum* species are among the well-tolerated plant species to environmental stress factors such as UV and soil. Although the levels of fatty acid compounds were detected at low levels in the extracts, they may contribute to biological activities such as reducing the risk of hypertension and cancer and lowering levels of serum cholesterol, triglycerides, and LDL cholesterol (Kim *et al*., 2014; Shen *et al*., 2012) as reported previously.

Table 1. GC-MS profile of *Helichrysum pallasii* leaves.

	Retention time	Compound	Relative concentration (%)		
			Ethanol	Infusion	Decoction
1	36.77	Palmitic acid	21.1±0.2^{c}	76.3±2.6^{a}	65.8±1.1^{b}
2	42.47	Linoleic acid	14.9±0.2^{a}	-	-
3	43.77	Linolenic acid	53.8±1.4^{a}	19.8±0.5^{c}	34.1±0.8^{b}

Means with different letters in the same column were significantly different at $p < 0.05$; all data were determined as a result of at least three independent experiments.

Figure 2. GC-MS profile of *Helichrysum pallasii* leaves



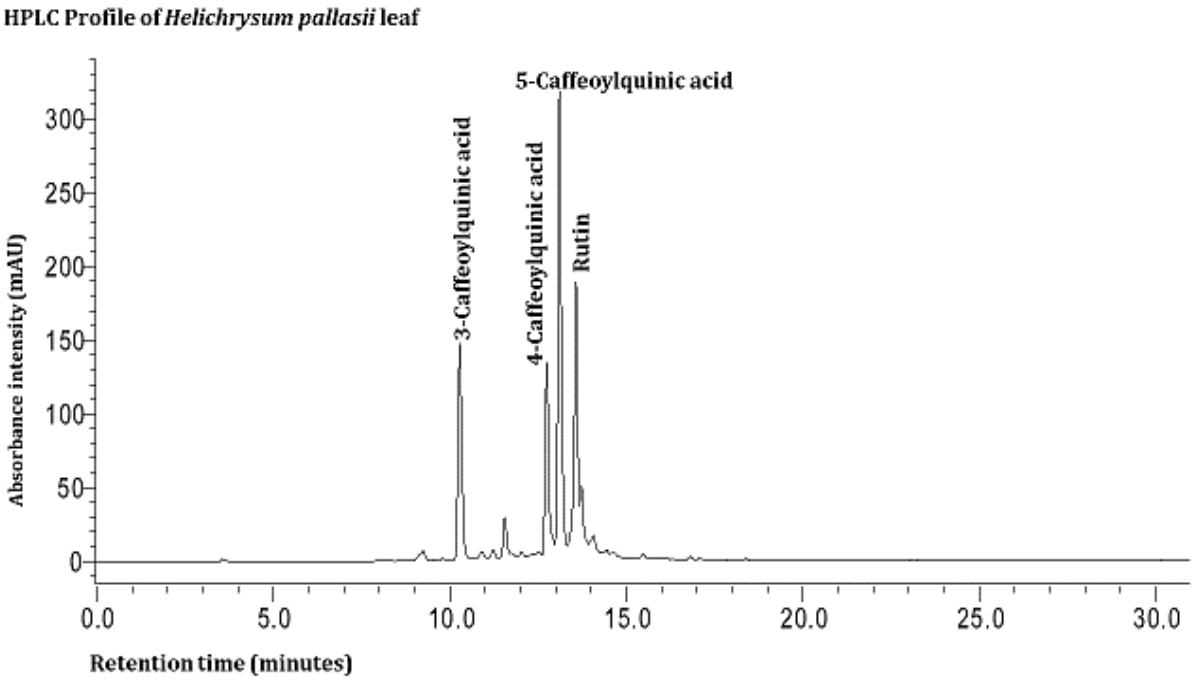
The identities of phenolic compounds were obtained using HPLC-PDA-MS/MS, and results are presented in Table 2 and Figure 3. As a result of the analysis, four compounds were determined. According to co-chromatography, UV spectrum properties, molecular weights, fragment ions, neutral loss, selective reaction imaging, and esterification reaction studies, the predominant compounds in the extracts were chlorogenic acid isomers (3-caffeoylquinic, 4-caffeoylquinic, and 5-caffeoylquinic acids) and the rutin.

Table 2. HPLC-MS/MS profile of *Helichrysum pallasii* leaf.

Individual phenolic compounds	MS/MS		Concentration (µg/mg extract)		
	-/[M-1]$^-$	Fragments (m/z) (+/-)	Ethanol	Infusion	Decoction
3-Caffeoylquinic acid	-/353	-/191, 179	14.9±0.2^a	9.1±0.1^b	7.5±0.1^c
4-Caffeoylquinic acid	-/353	-/191, 179, 173	9.2±0.1^a	5.2±0.1^b	4.7±0.1^c
5-Caffeoylquinic acid	-/353	-/191	42.5±1.2^a	25.8±0.9^b	19.9±1.0^c
Rutin	-/609	303/301	22.1±3.2^a	6.9±0.4^b	6.1±0.3^c

Means with different letters in the same raw were significantly different at the level ($p< 0.05$); n=3.

Figure 3. HPLC profile of *Helichrysum pallasii* leaf.



Various studies have been carried out on the beneficial effects of chlorogenic acid isomers and rutin until now. The most abundant form of chlorogenic acid in nature is 5-caffeoylquinic acid (Olthof *et al*., 2001). Chlorogenic acid isomers (3-caffeoylquinic, 4-caffeoylquinic, and 5-caffeoylquinic acids) show many biological activities such as antibacterial, antioxidant, anticarcinogenic, hypoglycaemic, hypolipidemic, hepatoprotective, antihistamine antimutagenic, anti-obesity, antidiabetic, antiviral, anti-Alzheimer, neuroprotective (Clifford *et al*., 2017; Hamed *et al*., 2020; Matthews *et al*., 2020; Torres *et al*., 2021). Rutin is a flavonoid found in many plants, also considered a non-toxic chemical that may be useful in biomedical applications and showing biological activities such as anti-inflammatory, anticarcinogenic,

antioxidant, neuroprotective, cytoprotective, vasoprotective, cardioprotective, and hepatoprotective (Ghorbani, 2017; Shahi *et al*., 2019).

The findings agree with the phytochemical composition results in species belonging to the *Helichrysum* genus. For example, Albayrak *et al.* (2008) reported the presence of p-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, hesperidin, apigenin-7-glucoside, naringenin, and apigenin compounds in the methanol extract prepared from the aerial part of *H. pallasii*. In other studies, significant compounds have been reported as chlorogenic acid, naringenin glucosides, quercetin, and apigenin glycosides in *H. stoechas* capitulum (Carini *et al*., 2001); apigenin, apigenin-7-glucoside, luteolin, naringenin and quercetin in *H. compactum* (Süzgeç *et al*., 2005); chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, syringic acid, apigenin, apigenin-7-glucoside, epicatechin, hesperidin, luteolin, naringenin and resveratrol in *H. arenarium* subsp. *erzincanicum*, *H. arenarium* subsp. *rubicundum*, *H. armenium* subsp. *araxinum*, and *H. plicatum* subsp. *pseudoplicatum* (Albayrak *et al*., 2010); p-hydroxybenzoic, caffeic acid, chlorogenic acid, 5,7-dihydroxy-3,6,8-trimethoxyflavone, isoquercitrin, quersetagetin-7-O glucopyranoside in the methanol extract of *Helichrysum stoechas* Moench plant (Les *et al*., 2017). The solvent structure of ethanol can explain the high levels of the phenolic compounds detected in the ethanol extract and its ability to extract rutin and chlorogenic acid compounds more effectively than other polar solvents (Naczk and Shahidi, 2006).

3.2. Biological Activities

3.2.1. *Antioxidant potential*

Oxidative stress can damage lipids, structural proteins, carbohydrates and the DNA chain, which occurs when the balance between free radicals and antioxidants is disrupted. Oxidative stress also constitutes the molecular basis for the occurrence of many degenerative disorders such as cancer, arterial diseases, neurodegeneration, diabetes, autoimmune disorders, rheumatoid arthritis, and kidney disease (Di Meo *et al*., 2016). Antioxidants play a significant role in the prevention of damage that may occur as a result of oxidative stress when the endogenous defence system in the organism is interrupted, or free radicals are produced in excessive amounts for various reasons, exceeding the capacity of the defence system (Andries *et al*., 2020; Pisoschi *et al*., 2021).

In this study, the antioxidant results were determined by Ferric reducing power (FRAP), Oxygen radical absorbance capacity (ORAC), DPPH radical scavenging activity, ABTS radical cation scavenging, Cupric ion reducing activity (CUPRAC), Phosphomolybdate total antioxidant capacity, and Metal chelation activity methods. Antioxidant capacity results and total phenolic contents of the extracts prepared in the leaf part of *Helichrysum pallasii* are presented in Table 3.

Table 3. Total phenolic contents and antioxidant activities of *Helichrysum pallasii* leaf.

7		*Helichrysum pallasii* leaf			Positive control (Synthetic antioxidants)			
		Ethanol	Infusion	Decoction	Ascorbic acid	Butylated hydroxyanisol	Trolox	Ethylenediamine tetraacetic acid
	Total phenolics [1]	103.0±5.1b	68.5±5.0c	51.8±1.8d	384±9a	-	-	-
Antioxidant activity	Ferric reducing antioxidant power [2]	2205.6±16.2b	1660.6±29.5c	1289.7±4.8d	5049±15a	-	-	-
	Oxygen radical absorbance capacity [3]	2540.5±42.5b	1800.7±42.2c	1278.9±6.9d	-	5991±74a	-	-
	DPPH radical scavenging activity [4]	0.58±0.01b	0.75±0.02c	0.87±0.19d	-	-	0.06±0.01a	-
	ABTS radical scavenging activity [4]	0.85±0.02b	1.14±0.03c	1.30±0.01d	-	-	0.18±0.02a	-
	Cupric ion reducing antioxidant capacity [4]	0.37±0.01b	0.56±0.01c	0.65±0.01d	-	-	0.11±0.01a	-
	Phosphomolybdenum total antioxidant capacity [4]	1.34±0.03b	1.61±0.02c	1.73±0.05d	-	-	0.55±0.1a	-
	Metal chelation activity [4]	1.42±0.02b	1.51±0.02c	1.72±0.07d	-	-	-	0.03±0.0a

Means with different letters in the same row were significantly different at the level ($p< 0.05$). All data represent the mean ± standard deviation of at least three independent experiments (n=3). [1] mg Gallic acid Equivalent/g extract, [2] µ mol Fe^{2+}/g extract, [3] µ mol Trolox Equivalent/g extract, [4] IC_{50}-half maximal inhibitory concentration (mg extract /ml).

Considering the obtained results, ethanol > infusion > decoction ranking was revealed in terms of antioxidative potential. Although the ethanol, infusion, and decoction extracts were found as effective antioxidant sources capable of both single electron and hydrogen atom transfer, as well as metal binding mechanisms. They showed weaker antioxidant activities than synthetic antioxidant agents such as gallic acid, butylated hydroxyanisole, trolox, and ethylenediamine tetraacetic acid, which were used as positive controls in the present study (Table 3).

It was determined that the total phenolic values of the extracts of *Helichrysum pallasii* leaves ranged between 51.8-103 mg Gallic acid/g extract. Albayrak *et al.* (2008) reported 94.1 mg GAE/g extract in methanol extract prepared from the aerial part of the *H. pallasii* plant. In another study, Albayrak *et al.* (2010) reported the total phenolic contents of methanol extracts prepared in the aerial parts of *H. arenarium* subsp. *erzincanicum, H. arenarium* subsp. *rubicundum, H. armenium* subsp. *Araxinum,* and *H. plicatum* subsp. *pseudoplicatum* plants at a range of 71.8-144.5 mg GAE/g extract. Also, a very similar total phenolic content (108.33 mg GAE/g extract) compatible with our finding was found by Özkan et al. (2004) in the methanolic extract of *H. chasmolycicum*.

The FRAP values of the extracts were determined in the range of 2205.6-1289.7 µmol Fe2+/g extract, and the highest FRAP value was found in the ethanol extract (Table 3). FRAP values of the *Helichrysum* genus used for treatment have been reported in various studies. For example, Zengin *et al.* (2020) the FRAP value of 80% above-ground methanol extract of *Helichrysum stoechas* plant as 662.87mg TE/g; Kolaylı *et al.* (2010) the FRAP value of the extract prepared in the leaf part of *H. plicatum* plant as 336.25 Trolox/ 100 g DW; Bojilov *et al.* (2019) the FRAP value of the ethanol extract of the aerial part of *H. italicum* as 106.92 mM TE/g DW were reported. Like FRAP results, ethanol extract had superior ORAC, DPPH, ABTS, CUPRAC, Phosphomolybdate total antioxidant, and metal chelation activities. The literature search (Albayrak *et al*., 2008; Albayrak *et al*., 2010; Kolaylı *et al*., 2010; Jahromi *et al*., 2017; Bojilov *et al*., 2019; Zengin *et al*., 2020) revealed similar DPPH (IC_{50} =26.23 µg/ml for *H.pallasii*; IC_{50} =23-39 µg/ml for *H. arenarium* subsp. *erzincanicum, H. arenarium* subsp. *rubicundum, H. armenium* subsp. *araxinum* and *H. plicatum* subsp. *pseudoplicatum*; IC_{50} =58 µg/ml for *H. plicatum* and IC_{50} =61 µg/ml for *H. leucocephalum*), ABTS (107.51 mM TE/g DW for *H. italicum*), CUPRAC (06.86 mM TE/g DW for *H. italicum* and 927.39 mM TE/g DW for *H. stoechas*), and phosphomolybdate (118.99 mg AAE/g extract for *H. pallasii*) results which are in agreement with our findings.

Considering all data obtained, ethanol solvent and low-temperature usage might provide higher levels of antioxidant phenolic compounds from plant matrix. Chlorogenic acid was one of the most capable antioxidant compounds among phenolics regarding hydrogen atom transfer mechanism (Ou *et al*., 2001), confirmed *in vitro* and *in vivo* (Liang and Kitts, 2016). Caffeoylquinic acid derivatives prevent many diseases with their protective roles against oxidative stress in the human body (Yoshimoto *et al*., 2002). The major phenolic compounds of *Helichrysum pallasii* identified within the present study, chlorogenic acid isomers and rutin, were reported as effective antioxidant agents.

3.2.2. *Enzyme inhibitory abilities*

Molecules that can partially or entirely bind to the target enzyme, slowing down or inhibiting its action, are called 'Enzyme Inhibitors'. Phytochemical compounds are efficient inhibitors as they are widely used to treat metabolic diseases. Active research areas of enzyme inhibitors are pharmaceutical chemistry, pharmacology, biochemistry and biotechnology, whose main goals are the design, discovery, and development of enzyme inhibitors (Bhagavan *et al*., 2015; Gonçalves and Romano, 2017; Patadiya *et al.*, 2021).

Enzyme inhibitory findings showed a similar pattern of activity (Table 4) which reveals ethanol solvent for the extraction of phenolic compounds that can bind enzyme protein. Scientific literature reports the superior inhibitory activity of ethanol extracts obtained from several *Helichrysum* species compared to water-based extracts (Orhan *et al.*, 2016; Gonçalves *et al.*, 2017). Water-based preparations (infusion and decoction) were found active only in pancreatic lipase inhibition. Total phenolic and individual phenolic concentration results positively correlate with suppressive enzyme activities.

Table 4. Enzyme inhibitory activities of *Helichrysum pallasii* leaf.

			Ethanol	Infusion	Decoction
Enzyme inhibitory activity	Acetylcholinesterase	IC_{50} (mg/ml)	1.49±0.10	NA	NA
		Galanthamine Equivalent (mg/g extract) *	1.98±0.11	NA	NA
	Butyrylcholinesterase	IC_{50} (mg/ml)	1.66±0.26	NA	NA
		Galanthamine Equivalent (mg/g extract) *	1.86±0.46	NA	NA
	Tyrosinase	IC_{50} (mg/ml)	0.68±0.01	≥3	≥3
		Kojic acid Equivalent (mg/g extract) *	123.99±0.24	12.45±2.78	12.09±2.32
	Alpha-Amylase	IC_{50} (mg/ml)	2.09±0.24	≥3	≥3
		Acarbose Equivalent (mmol/g extract) *	0.51±0.07	0.07±0.01	0.08±0.01
	Alpha-glucosidase	IC_{50} (mg/ml)	0.51±0.01	≥3	≥3
		Acarbose Equivalent (mmol/g extract) *	11.7±0.06	1.33±0.05	1.11±0.23
	Pancreatic Lipase	IC_{50} (µg/ml)	42.5±4.2	56.8±3.9	62.1±3.4
		Orlistat Equivalent (µmol/g extract) *	88.6±5.1	67.2±2.1	59.1±2.1

Means with different letters in the same row were significantly different at the level ($p< 0.05$); All data represent the mean ± standard deviation of at least three independent experiments (n=3). NA: not active (no inhibition was observed). *The equivalent of commercial standards calculated based on a standard curve and against control.

Ethanol extract containing a high amount of antioxidant phenolic compounds may be a candidate for biotherapeutic agents that can be used for antidiabetic purposes due to its low alpha-amylase and high alpha-glucosidase activities, which can minimize the accumulation of undigested carbohydrates in the large intestine (Exteberria *et al*., 2012). Obesity is a disease that occurs with the accumulation of abnormal or excessive fat in the body in a way that impairs health and negatively affects the quality of life, which is associated with diabetes, cardiovascular diseases, gastrointestinal system diseases and some types of cancer (Weiss *et al.*, 2013). Phenolic-rich herbal extracts were reported as promising candidates for anti-obesity agents whose mechanism is based on inhibitory of pancreatic lipase *in vitro* and *in vivo* (Cho *et al*., 2010; Karthikesan *et al*., 2010; Dalar *et al*., 2014; Zhang *et al*., 2015).

The dominant phenolic compounds of the *Helichrysum pallasii* extracts were chlorogenic acid isomers which were previously reported as potent antidiabetic agents in vitro (α-glucosidase; IC_{50}: 9.1 µg/ml and α-amylase; IC_{50}: 9.2 µg/ml) (Oboh *et al*., 2015) and *in vivo* (Jung *et al*., 2006; Kamalakkannan and Prince, 2006; Ong *et al*., 2012; Torres *et al.*, 2021).

The primary mode of inhibition determined for the digestive enzymes of polyphenolic compounds is the non-competitive mode. The structure of proteins plays an essential role in protein-phenolic compound interaction. Therefore, the formation of protein-phenolic compound complexes that inhibit enzyme activity depends on the molecular structure of a

phenolic compound and on the amino acids that make up the protein structure. For instance, pancreatic lipase mainly comprises four pancreatic lipase amino acids binding sites such as Phe^{78}, Tyr^{115}, His^{152} and Phe^{216}, for binding polyphenolic compounds (Cho *et al*., 2010; Martinez-Gonzalez *et al*., 2017). Hu *et al.* (2015) reported that caffeoylquinic acid isomers could effectively bind the sites of His^{263}, Asp^{176} and Ser^{15}, which indicate the high lipase inhibitory activities of *H. pallasii* extracts.

4. CONCLUSION

The phytochemical composition and biological activities of traditional preparation methods (infusion and decoction) and ethanol-based extract prepared from *H. pallasii* leaves were assessed. The ethanol-based extract had higher phenolic fatty acid content and antioxidant and enzyme suppressive activities. The phenolic composition comprised chlorogenic acid isomers (3-caffeoylquinic, 4-caffeoylquinic, and 5-caffeoylquinic acids) and the rutin. The lipophilic composition consisted of fatty acids, including palmitic, linoleic, and linolenic acids. These findings contribute to the scientific literature on developing and investigating biotherapeutic agents that can be used in pharmaceutical and therapeutic industries.

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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

Nejmi Isik: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Muzaffer Mukemre**: Methodology, Investigation. **Rabia Sena Turker**: Methodology, Investigation. **Gokhan Zengin**: Methodology, Investigation. **Abdullah Dalar**: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft.

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Research Article

Assessment of genetic diversity based on agro-morphological traits and ISSR molecular markers in einkorn wheat (*Triticum monococcum* ssp. *monococcum*) landrace populations from Turkey

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Abstract: The aim of this study is to investigate genetic diversity in 48 einkorn (*Triticum monococcum* L. ssp. *monococcum*) landraces grown in agricultural areas of Bolu and Kastamonu. Therefore, variation in seven agro-morphological traits was investigated. Agro-morphological traits such as leaf weight (mg), coleoptile length (cm), root number (n), root length (cm), fresh root weight (mg), and dry root weight (mg) were examined by the coefficient of variation, ANOVA, and principal components analysis. The highest coefficient of variation (%) was observed in fresh root weight (FRW = 52.09%), while the lowest was in leaf weight (LW = 8.9%). Principal Component Analysis (PCA) was calculated as 76.93% variation in two main components. For molecular characterization, data obtained with iSSR primers were analyzed with the population genetics analysis program PopGene (ver. 1.32). According to PopGene results, the mean number of alleles, the mean number of effective alleles, and average genetic diversity values were calculated as na = 2, nea = 1.33, and h = 0.13, respectively. Among the agro-morphological traits, germination power, root number, and coleoptile length appeared to be reliable traits. The results show that the use of morphological characters alone for genetic diversity in populations is not sufficient to determine the difference between populations and their genetic structure.

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1. INTRODUCTION

The *Triticum* genus is divided into three groups according to the number of chromosomes: diploids (*T. monococcum* L. ssp. *monococcum* 2n = 14, *AA*), tetraploids (*T. turgidum* L. 2n = 28, *AABB*), and hexaploids (*T. aestivum* L. 2n = 42, *AABBDD*). The name "einkorn," a single grain, comes from Germany. It is locally named "Iza" or "Siyez" in Türkiye. Einkorn spikes have single-grain and a husky structure (Aslan *et al*., 2018; Karagöz & Zencirci, 2005; Ünlü *et al*., 2018).

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In Türkiye, especially in the Western Black Sea Region (Sinop, Kastamonu, Samsun, and Bolu), *Triticum monococcum* L. ssp. *monococcum* is called IZA (einkorn) wheat and is consumed mostly as bulgur. There are 1.400 hectares of einkorn wheat cultivation in the Kastamonu region. While the production amount of einkorn wheat throughout Kastamonu is 3,500 tons, according to the data from the İhsangazi District Directorate of Food, Agriculture, and Livestock, İhsangazi had the highest production rate in 2013 with 6.750 decares, an einkorn wheat production of 1.687 tons, and an einkorn bulgur production of 470 tons. There are 1,050 Einkorn wheat farmers in the district, and the Einkorn Wheat Growers Association, which was established in İhsangazi, has approximately 100 members (Aslan *et al*., 2018; Ünal, 2002). Production in Seben and Bolu has also increased recently.

Løje *et al*. (2003) observed that ten Einkorn populations had a large amount of ash (2.3-2.8% DM), a variable level of proteins (10.03-26% DM), and glucan (0.29-0.71 % DM). It has a very low level of nutritional fiber (7.6 –9.9% KM), highly variable lysine levels (1.51–3.15 gram lysine 100 g^{-1} protein), low sedimentation, and a thin mixographer's curve compared to typical wheat cultivars (Abdel-Aal *et al*., 1997). The protein content is generally greater than that of rye and hard red wheat. The structure of amino acids is similar to common wheat (Troccoli & Codianni, 2005).

In the study of Abdel *et al*. (1997), the results show that einkorn has a high protein content but low gluten elongation ability. Cooked einkorn grains have a softer consistency, less white color, less stickiness, and a less fibrous structure than those of *spelta* and common wheat (Bavec & Bavec, 2006).

Based on previous studies, Zencirci *et al*. (2018) stated that the number of botanical varieties grown in Türkiye with all *Triticum* ssp. cultivated in Türkiye exceeds substantially the number of botanical wheat varieties cultivated in the other parts of the world. For instance, of the 73 botanical *T. turgidum* varieties recognized at the time, 48 were collected from Türkiye.

Guzy *et al*. (1989) identified a wide diversity in the number of spikelets per spike and the number of grains per spike in a series of diploids, tetraploids, and hexaploids. Sharma *et al*. (1984) compared 93 genotypes of einkorn entries with "Modoc" durum wheat and "Anza" bread wheat varieties. They compared several parameters, such as plant height (PH), grain weight, lysine content and protein ratio in flour, ear weight, earliness, height, etc., and they observed a wide genetic variation among the samples. Castagna *et al*. (1995) studied 21 *Triticum monococcum* L. ssp. *monococcum* populations from different locations and found that there were important genetic variations for ear emerging date, plant height (PH), grain yield, total biomass, and the number of ears per m^2. Empilli *et al*. (2000) examined 1,039 einkorn genotypes and reported that there was a wide variation for grain size, 13 genotypes with higher thousand kernel weight over 40 g, many genotypes with low SDS sedimentation, and eight genotypes with higher SDS sedimentation. Butnaru *et al*. (2003) characterized 37 local einkorn wheat genotypes collected from Romania and Hungary for six morphological characters and five agronomic characteristics and found that the number of seeds per spike and grain weight was diverse among genotypes. In the study of Özbek and Zencirci (2021), six einkorn (*Triticum monococcum* L. ssp. *monococcum*) landrace populations and two bread wheat (*Triticum aestivum* L.) populations were collected from agricultural areas in Bolu and Kastamonu provinces. They were characterized for genetic diversity by using 12 Intel simple sequence repeat primers. The genetic diversity was observed to be $h = 0.20$ in *T. monococcum* populations and $h = 0.14$ in *Triticum aestivum* populations. A dendrogram was constructed according to the genetic distance values by using the unweighted pair group method with the arithmetic mean method. *Triticum aestivum* and *Triticum monococcum* populations are clustered into different clusters (Zencirci *et al*. 2019). The number of sub-populations was

identified as the optimal value for $K = 7$. ISSR markers were successful in determining the genetic diversity and population structure within and between species.

This study aims to investigate the agro-morphologic and molecular characters in einkorn wheat (*Triticum monococcum* L. ssp. *monococcum*) from Türkiye. We investigated the agro-morphological characters in einkorn wheat, such as germinating power (GP), coleoptile length (CL), root count (RC), root length (RL), root fresh weight (RFW), root dry weight (RDW), and leaf weight (LW) in addition to the variation in molecular iSSR markers. The possibility of the development of more superior wheat varieties can be increased by determining the agro-morphological and molecular characteristics of wheat.

2. MATERIAL and METHODS

2.1. Materials

In this study, agro-morphological traits were investigated to determine the genetic diversity in 48 einkorn wheat (*Triticum monococcum* L. ssp. *monococcum*) local populations from Türkiye (Table 1). The seed samples were collected from the agricultural areas in İhsangazi (Kastamonu) and Seben (Bolu) in 2020.

Table 1. Using Einkorn populations in the study (abbreviations: population ID number N, registration number RN, and local name LN)

N	RN	Species Name	LN	Collection sites
1	10	*T. monococcum* ssp. *monococcum*	Einkorn	Kastamonu/Ihsangazi/ Uzunoğlu Mah.
2	11	*T. monococcum* ssp. *monococcum*	Einkorn	Kastamonu / İhsangazi / ÇayMah.
3	14	*T. monococcum* ssp. *monococcum*	Einkorn	Kastamonu / İhsangazi.
4	16	*T. monococcum* ssp. *monococcum*	Einkorn	Kastamonu / İhsangazi / Koçcugaz Köyü
5	29	*T. monococcum* ssp. *monococcum*	Einkorn	Kastamonu / Araç / Aliören Köyü
6	35	*T. monococcum* ssp. *monococcum*	IZA	Bolu / Seben / Musasofular Köyü
7	37	*T. monococcum* ssp. *monococcum*	IZA	Bolu / Seben / Musasofular Köyü Çıkışı
8	39	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/GüneyceKöyü.
9	44	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/YakuplarKöyü/Aynak Deresi Mevkii
10	43	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/ YakuplarKöyü.
11	45	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/YakuplarKöyü/Aynak Deresi Mevkii
12	47	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Musasofular Köyü.
13	48	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Haccağız Köyü/BeylikMevkii
14	49	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Musasofular Köyü
15	50	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Gerenözü Köyü
16	51	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Nimetli Köy
17	54	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Gerenözü Köyü
18	55	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Nimetli Köyü
19	56	*T. monococcum* ssp. *monococcum*	IZA	Bolu/HaccağızKöyü/Beylik Mah
20	57	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Musasofular Köyü/Akcumar Bölgesi
21	58	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
22	59	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
23	60	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
24	B24	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
25	B17	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
26	B35	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
27	B73	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Çaylak Köyü
28	B63	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Aşağı Kınık Köyü

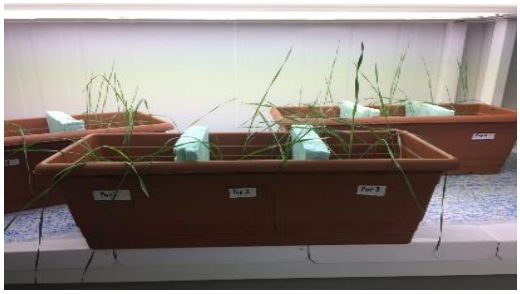
Table 1. *Continues.*

29	B71	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Sarılar Köyü
30	B78	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Aşağı Kınık Köyü
31	B61	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Yukarı Kınık Köyü
32	B64	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Yukarı Kınık Köyü
33	B69	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Yukarı Kınık Köyü
34	B20	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
35	B26	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Haccağız Köyü
36	B32	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Musasofular Köyü
37	B30	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Değirmenkaya Köy
38	B66	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Yukarı Kınık Köyü.
39	B25	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
40	B21	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Yağma Köyü
41	B65	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Göynük/Pelitçik Köyü
42	B33	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
43	B19	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
44	B18	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
45	B16	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
46	B23	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Yağma Köyü
47	B22	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü
48	B15	*T. monococcum* ssp. *monococcum*	IZA	Bolu/Seben/Güneyce Köyü

2.2. Methods

The agro-morphological traits were determined for germination power (GP), coleoptile length (CL), number of roots (NR), root length (RL), root fresh weight (RFW), root dry weight (RDW), and leaf weight (LW). Five seed samples from each population were planted in the soil and sterilized in the autoclave for 15 minutes at a temperature of 121 °C. Then, they were placed in the climate chamber, which was kept at constant climate conditions (Temperature 23° C ± 2, 16 hours day, 8 hours night) for 30 days. After 30 days, the plants were cut, and the leaves were weighed. The leaves were kept at -20 °C for molecular analyses of the populations (Figure 1).

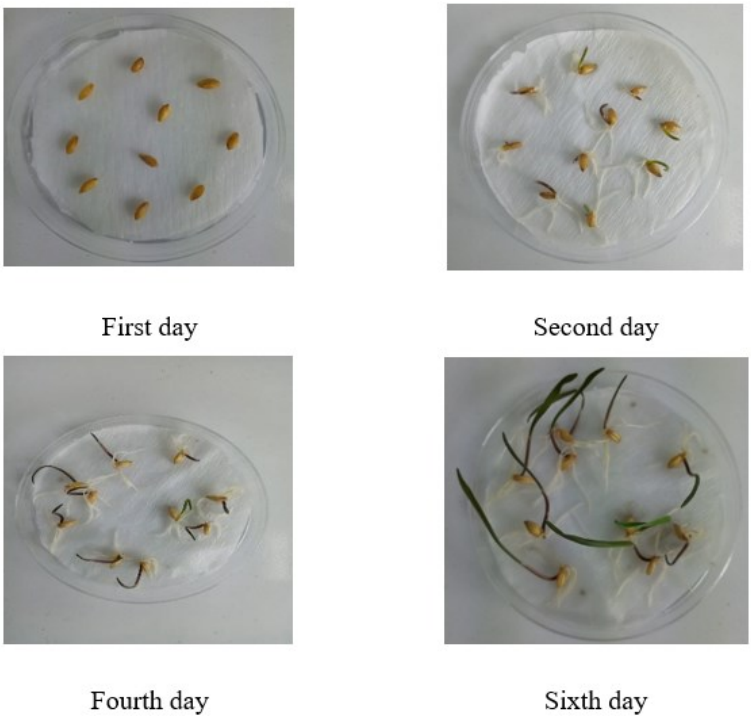
Figure 1. After 30 days, the plants.



The husks of 50 seeds in each population were peeled and cleaned for the measurement of germination power, coleoptile length, number of roots, root length, root fresh weight, and root dry weight. Then, the seeds of each population (50 seeds) were sterilized with the disinfection solution, which was prepared with two drops of Tween 20, 30 ml of bleach, and 70 ml of distilled water in total in a 500-ml glass beaker for 10 minutes. The floated seeds were then removed because they were considered dead seeds. Sterile conditions were achieved by placing three Petri plates, three filter papers, and 500 ml of water in a glass tube with a total volume of 1000 ml in a sterile room. Next, the sterilized seeds were placed in three Petri dishes in which

two filter papers were placed and moistened with 2 ml of water in the form of ten seeds. After that, the plates were placed inside the climate chamber with a constant climate condition (23±2˚C for 16 hours during the day and 8 hours at night). The germination process, which involved moistening each petri dish with 2 mL of water every day, took place after 8 days (Figure 2).

Figure 2. The images show the stages of the germination process between 1st-6th days.



After eight days from germination (Figure 3), the plantlets were cut, and data were taken for GP, CL, RN, RL, FRW, and DRW. The roots were cut, weighed in their fresh state, put in the incubator at 37 °C for four days, then weighed in their dry state.

Figure 3. After eight days of germination, the seed is harvested.

The aims of the molecular character's experiment were (i) to investigate genetic diversity in *Triticum monococcum* ssp. *monococcum* landrace populations, (ii) to investigate genetic diversity in *Triticum monococcum* ssp. *monococcum* landrace sub-populations, and (iii) to determine population structure and genetic differentiation among the populations and among the sub-populations in *Triticum monococcum* ssp. *monococcum* landrace populations grown in farmers' fields in Bolu and Kastamonu provinces in Türkiye.

DNA Isolation: The genomic DNA was extracted from the leaves of 1-1.5-month-old plants by the modification method as defined by Kidwell and Osborn (1992) as follows:

1. Collect more than one gramme of young leaf tissue from wholesome plants, freeze the samples, and keep at -20 °C in the desiccator.
2. Melt 250-300 mg lyophilized tissue into fine powder at room temperature and transfer tissue to the marked polypropylene pipe of 15 or 50 ml.
3. Add DNA buffer (5-10 ml) (approximately 1 ml per 30-50 mg tissue; the optimal ratio of tissue may vary with different plant species). Suspend the tissue in the buffer carefully and completely with gentle, rocking movements.
4. Incube with periodic mixing for 60 min at 55-60°C.
5. Add chloroform and isoamyl alcohol in equal proportions (24:1) and carefully and gently mix together. 1000-5000 g centrifuge, 20 ° C for 30-50 minutes.
6. Put the aqueous (upper) phase into the marked 50-ml tube using a large pipette and add 2,5 EtOH volumes to the tube (-20 °C) or 0,6-1 isopropanol volumes (-20 DC). Mix well until DNA rushes.
7. Choose the suitable procedure for washing, drying, and re-dissolving samples according to the state of precipitated DNA.

DNA Amplification: For DNA amplification, iSSR-PCR reactions were performed in a volume of 20 µL reaction mixture containing 1x Taq buffer (10×), 3 mM MgCl2 (25 mM), 200 µM dNTPs (10 mM each), 0.2 u of Taq DNA polymerase (5u/µL, Thermo), 0.2 µMISSR primer (10 pMol, Query, Alpha DNA), 1 µL template DNA (10–40 ng) in final concentration and distilled water was added up to 20 µL. PCR amplification was carried in a Thermo Scientific thermocycler PCR system.

The thermal program for DNA amplification was programmed as one cycle for 4 min at 94°C, 35 cycles for 45 s at 94°C, for 30 s at 58°C, and for 2 min at 72°C, followed by one cycle for 7 min at 72°C. The iSSR-PCR amplicons were run along with a 100-bp DNA molecular size marker (Thermo) on a 1.3% agarose gel (Sigma), and electrophoresis was carried out at 80 mA / 160 V for 2-2.5 h. Ethidium bromide (10 mg/mL) staining was used to visualize amplified fragments, and the pictures were taken under UV light (DNR bio-imaging system).

2.3. Statistical Analysis

2.3.1. *The Kaiser-Meyer-Olkin (KMO) test:*

To analyze the data on agro-morphological traits in each population, the Kaiser-Meyer-Olkin analysis (KMO) was applied to compare the populations. The KMO test is a metric test to estimate whether the information is suitable for factor analysis. The statistics were used to quantify the proportion of the difference between variables that is considered normal. The lower the percentage, the better the factor analysis (Goto *et al*., 2011).

The variance displayed the proportion of the deviation (dispersion) of the data collected by a statistical model. Often, a deviation is measured as a variance, so the explicit variance is used for more precise expression. The difference in fraction described by the main component is the ratio of the variance of the main component to the total variance (O'Grady, 1982). The estimate of the sample variances for all the individual variables is called the total variance (O'Grady, 1982). Bartlett's sphericity test, often performed before PCA or factor analysis, examines whether data come from a multivariate standard zero covariant distribution (Jackson, 1993).

2.3.2. *Coefficient of variation % (CV %)*

The coefficient of variation indicates the degree of variability in data as relative to the population mean. The CV % is the ratio of the standard deviation to the mean. A high coefficient of variation means a high distribution around the mean. Without units, measurement scales

allow comparison between distributions of non-comparable values. The lower the value of the coefficient of variation, the more accurate the prediction (Insee, 2016). If CV values are categorized according to ranges; CV<10 is very good, 10-20 is good, 20-30 is acceptable, and CV>30 is not acceptable (Insee, 2016).

2.3.3. *Analysis of variance (ANOVA)*

ANOVA is a type of statistical test used to determine if there is a statistically significant difference between two or more categorical groups by testing for differences in means using variance. Two hypotheses are proposed, in the null hypothesis (H_0), and the difference between the means of the groups is statistically significant. In the alternate hypothesis (H_0), when $p< \alpha$ (0.05) is calculated, it means that the means of some of the groups are unequal, and H_0 is rejected according to Steel and Torrie (1980).

2.3.4. *Principal component analysis (PCA)*

The principal component analysis distills the essence of the data into a few key components that explain the most variation in the data set. The principal components, which are based on the eigenvectors of the correlation matrix derived from the boron treatment data set of 48 einkorn wheat genotypes, were calculated by IBM-SPSS statistical software.

2.3.5. *Pearson's correlation*

The Pearson's correlation values were observed among the agro-morphological traits in einkorn wheat landraces populations. Pearson's correlation coefficients (r_P) were computed to relate the measures of metric agro-morphological traits by using SPSS statistical software (version 22 for Windows) according to Steel and Torrie (1980).

3. FINDINGS

3.1. Descriptive Statistics

According to descriptive statistics, the minimum values for agro-morphological traits ranged between 0.37 and 74.48 for the traits RL and LW, respectively, while the maximum values ranged between 1.70 and 426.90 for the traits CL and LW, respectively. The highest mean value was observed as 215.16 for LW, while the lowest mean value was observed as 1.06 for CL (Table 2). The standard deviation range was calculated between 0.30 and 68.89.

Table 2. The descriptive values observed in agro-morphological traits in Einkorn wheat landrace populations (abbreviations: N: Sample Number, CL: Coleoptile Length, RN: Root Number, RL: Root Length, GP: Germination Power, FRW: Fresh Root Weight, DRW: Dry Root Weight, LW: Leaf Weight)

	N	Minimum	Maximum	Mean	Std. Deviation
CL	48	0.40	1.70	1.06	0.30
RN	48	1.27	4.53	3.20	0.74
RL	48	0.37	3.41	1.75	0.67
GP	48	5.33	10.00	8.98	0.81
FRW	48	9.37	141.60	60.50	31.85
DRW	48	2.23	35.17	15.45	8.00
LW	48	74.48	426.90	215.16	68.89

3.2. Coefficient of Variation

The mean values for agro-morphological traits ranged between 1.06 and 215.16. According to the coefficient of variation values, the highest value was observed in FRW at 52.09%, while the lowest value was observed in LW at 8.9% (Table 3).

Table 3. Coefficient of variation in agro-morphological traits calculated in einkorn wheat populations (abbreviations: CL: Coleoptile Length, RN: Root Number, RL: Root Length, GP: Germination Power, FRW: Fresh Root Weight, DRW: Dry Root Weight, LW: Leaf Weight, N: Sample Number, M: Mean, SS: Squared Deviation, CV: Coefficient of Variation).

	N	M	SS	$\sigma^2 = SS/N$	$\sigma = \sqrt{\sigma^2}$	CV (%) = (σ/M) *100
CL	48	1.06	4.31	0.09	0.3	28.26
RN	48	3.2	26.06	0.54	0.74	23.02
RL	48	1.75	20.84	0.43	0.66	37.71
GP	48	8.98	31.03	0.65	0.80	8.95
FRW	48	60.50	47672.91	993.19	31.51	52.09
DRW	48	15.45	3007.20	62.65	7.92	51.23
LW	48	215.16	223075.05	4647.4	68.17	31.68

3.2.1. *One-way ANOVA*

According to one-way ANOVA, the measurement of agro-morphological traits of 48 *Triticum monococcum* L. ssp. *monococcum* landrace populations differed significantly between the groups; thus, the null hypothesis was rejected. The F values were 307.16 (at $p<0.05$ significance level) between the groups. When the results of the ANOVA were significant for the agro-morphological traits of 48 *Triticum monococcum* L. ssp. *monococcum* landrace populations, a post hoc test-Tukey's HSD was run to identify where the differences truly came from.

According to Tukey's HSD, the measurement of agro-morphological traits in 48 *Triticum monococcum* L. ssp. *monococcum* landrace populations was not homogeneous, and significant variations were determined between the groups. On the other hand, the variations within the groups were not significant. Significant differences were observed between FRW and CL, RN, RL, GP, and DRW as 60.04, 57.91, 59.35, 52.17, and 45.52, (at $p<0.05$ significance level), respectively, while between LW and CL, RN, RL, GP, FRW, and DRW, significant differences were observed as 215.52, 213.39, 214.83, 207.66, 155.48, and 201.00 (at $p<0.05$ significance level), respectively.

3.3. Pearson's Correlations among the Agro-Morphological Traits

The pearson's correlations among the agro-morphological traits were performed. The results indicated that there were significant correlations among the traits. The highest significant value was determined as 0.910 ($r = 0.00$ at $p <0.01$ significant level) between DRW and RL, while the lowest significant value was 0.296 ($r = 0.04$ at $p <0.05$ significant level) between LW and RL (Table 4).

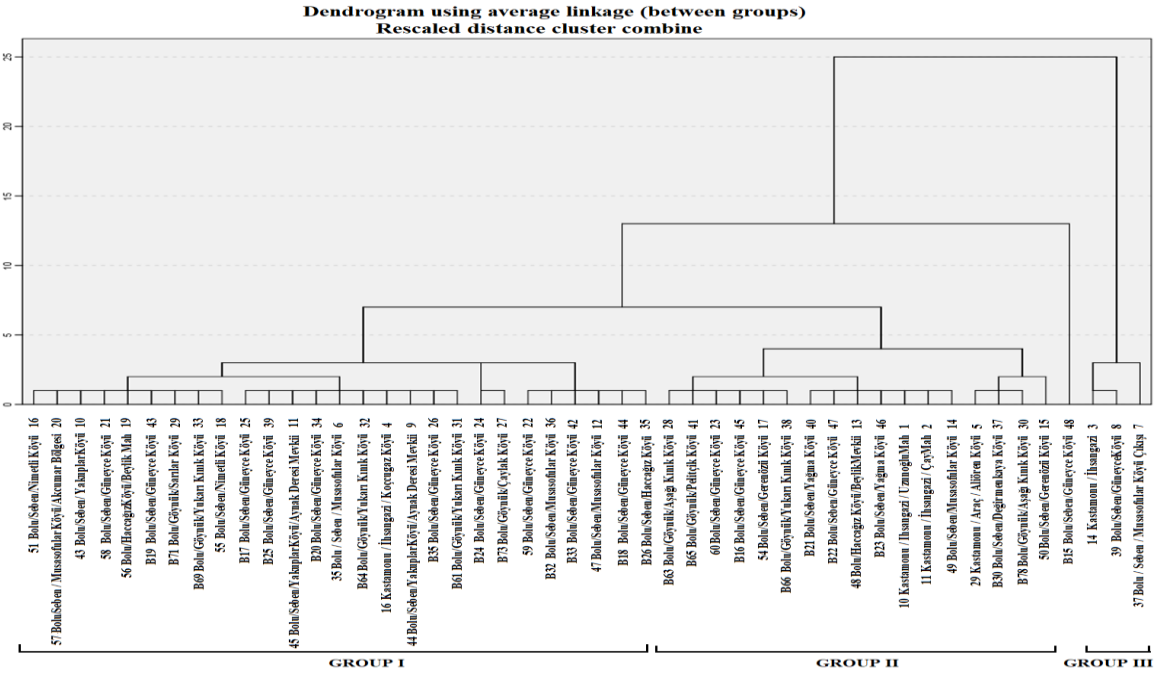
Table 4. Coefficient of variation in agro-morphological traits calculated in einkorn wheat populations (abbreviations: CL: Coleoptile Length, RN: Root Number, RL: Root Length, GP: Germination Power, FRW: Fresh Root Weight, DRW: Dry Root Weight, LW: Leaf Weight, N: Sample Number, M: Mean, SS: Squared Deviation, CV: Coefficient of Variation).

		CL	RN	RL	GP	FRW	DRW	LW
CL	r_P	1.00	-	-	-	-	-	-
	p	-	-	-	-	-	-	-
RN	r_P	0.815**	1.00	-	-	-	-	-
	p	0.00	-	-	-	-	-	-
RL	r_P	0.629**	0.695**	1.00	-	-	-	-
	p	0.00	0.00	-	-	-	-	-
GP	r_P	0.438**	0.561**	0.14	1.00	-	-	-
	p	0.00	0.00	0.33	-	-	-	-
FRW	r_P	0.356*	0.538**	0.870**	0.01	1.00	-	-
	p	0.01	0.00	0.00	0.92	-	-	-
DRW	r_P	0.571**	0.715**	**0.910****	0.13	0.832**	1.00	-
	p	0.00	0.00	0.00	0.39	0.00	-	-
LW	r_P	0.04	0.20	**0.296***	0.10	0.28	0.405**	1.00
	p	0.80	0.17	0.04	0.50	0.05	0.00	-
N		48	48	48	48	48	48	48

3.4. The Phylogenetic Relationships Among All Analyzed Populations According to Agro-Morphological Characters

A dendogram was constructed based on agro-morphological traits. The Einkorn wheat landrace populations were clustered into two main groups (Figure 4). The populations from the same area tended to be grouped into the same sub-groups in the first main group I. In the second main group, only three populations, one from Kastamonu and two from Bolu Provinces, were grouped as an out-group.

Figure 4. The dendrogram was constructed according to the between-group linkage method based on squared Euclidean distances, representing the phylogenetic relationship between 48 Turkish Einkorn wheat landrace populations.



3.5. Principal Component Analysis (PCA)

According to PCA results, the first two eigenvalues explain about 76.93% of the variance in the seven-dimensional data for morphometric data (Table 5). The first PC is the linear combination PC1 = 0.76 CL + 0.88 RN + 0.93 RL + 0.81 FRW + 0.92 DRW and 0.37 LW. It can be interpreted as a contrast between the CL and RN variables and the RL, FRW, DRW, and LW variables. For the second PC, the coefficients for the GP variable were small, PC2 = + 0.77 GP (Table 6). It can be interpreted as a weighted sum of vectors that point mostly in the direction of the GP, CL, and RN. In the component plot graph, the horizontal axis represents the PC1, and the vertical axis represents the PC2 (Figure 5).

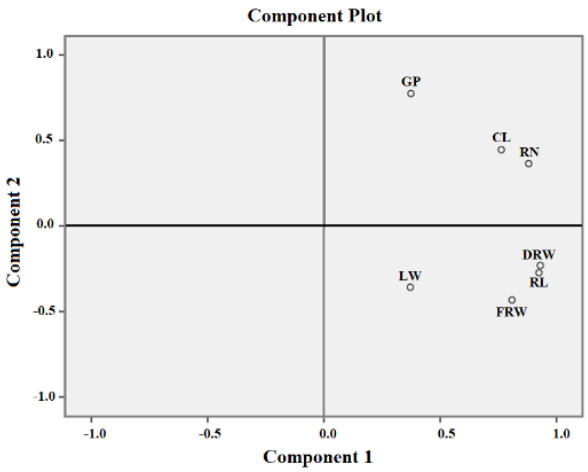
Table 5. Total variance explained by principal component analysis (PCA) performed by using data from CL, RN, RL, GP, FRW, DRW, and LW as variables according to the *Pearson* correlation (one-tailed) matrix with Eigenvalues, percentage of variance, and cumulative percentage of variance (C: component)

C	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	4.01	57.29	57.29	4.01	57.29	57.29
2	1.37	19.64	76.93	1.37	19.64	76.93
3	0.91	13.04	89.97			
4	0.40	5.75	95.73			
5	0.14	2.05	97.78			
6	0.10	1.47	99.25			
7	0.05	0.75	100.00			

Table 6. Component matrix of values, produced by PCA, of the variables (CL, RN, RL, GP, FRW, DRW, and LW) and their contribution to principal components (abbreviations: V: Variable, CL: Cleoptile Length, RN: Root Number, RL: Root Length, GP: Germination Power, FRW: Fresh Root Weight, DRW: Dry Root Weight, LW: Leaf Weight).

V	Components	
	1	2
CL	0.76	0.45
RN	0.88	0.36
RL	0.93	-0.23
GP	0.37	0.77
FRW	0.81	-0.43
DRW	0.92	-0.27
LW	0.37	-0.36

Figure 5. The component plot graph constructed by PCA represents the component 1 and component 2 values derived from 48 einkorn wheat landrace populations (abbreviations: CL: Cleoptile Length, RN: Root Number, RL: Root Length, GP: Germination Power, FRW: Fresh Root Weight, DRW: Dry Root Weight, LW: Leaf Weight)



3.6. The Statistical Analysis for Population Genetics

The iSSR-PCR amplification fragments were scored using TotalLab Image Quant software along with visual scores on the photographs of the gels. The raw information was translated into binary data: 1 for the current fragment and 0 for the missing fragment. Therefore, the data were diploid and dominant, and binary data were calculated using PopGen version 1.32 for population genetic analysis (Yeh *et al*., 1997). The mean number of alleles per locus (n_a), efficient alleles per locus (n_{ea}), and the mean value of genetic diversity (h) have been estimated for gene diversity estimates (Nei, 1973). The genetic distinction between populations was normally calculated by G_{ST} for mostly inherited DNA markers (Nei, 1973), which demonstrates the separation of genetic differences within and within populations. The gene flow (N_m) between populations of the sample was calculated using the G_{ST}value. Dendrograms built using an unweighted pair group approach with an arithmetic average (UPGMA) based on iSSR data were used to represent the phylogenetic relationships between populations or subpopulations.

3.6.1. *Genetic diversity estimates of einkorn wheat landrace populations*

The genetic diversity of einkorn wheat (*Triticum monococcum* L. ssp. *monococcum*) landrace populations was investigated by the inter-simple sequence repeats (iSSR) molecular marker system. In this study, 10 x 3 seeds for each population seed were analyzed using the iSSR primer UBC-826, which produced 30 polymorphic bands. The mean number of alleles, effective allele, and genetic diversity value at the locus level were observed to be 2, 1.33, and 0.13, respectively (Table 7). The highest number of alleles existed in Bolu-Seben population, while the lowest number was in Kastamonu-İhsangazi population. The highest number of effective alleles and genetic diversity values were observed as 1.29 and 0.18 in both Bolu-Seben / Güneyce and Bolu - Seben populations, respectively. The highest number of polymorphic bands was determined to be 22 (73%) in the Bolu-Seben population, while the lowest polymorphic band was determined to be 15 (50%) in the Kastamonu-İhsangazi population.

The total genetic diversity and the genetic diversity within the populations were identified as 0.21 and 0.17 at population levels, respectively (Table 8). The genetic differentiation among the populations was calculated at 0.21, while the gene flow between the populations was 1.91. The genetic distance between Population 1 and both Populations 2 and 3 was 0.06, while the genetic distance between Populations 2 and 3 was 0.12 (Table 9). Using iSSR data, the researchers were able to generate a dendrogram showing how different populations clustered. As a result, the Bolu population clustered away from the Kastamonu population (Figure 6).

Table 7. The total genetic diversity estimates among the Einkorn wheat landrace populations.

POP	Sample Size	n_a	n_{ea}	h	# PL	% PL
BOLU-SEBEN/GÜNEYCE	10	1.67	**1.29**	**0.18**	20	0.67
BOLU-SEBEN	10	**1.73**	**1.29**	**0.18**	**22**	**0.73**
KASTAMONU-İHSANGAZİ	10	**1.50**	**1.22**	**0.13**	**15**	**0.50**
MEAN	30	2	1.33	0.21	30	100

Table 8. The total genetic diversity and F statistics estimates among the Einkorn wheat landrace populations.

	Sample Size	H_T	H_s	G_{ST}	N_m
MEAN	30.00	0.21	0.17	0.21	1.91

Table 9. The genetic distance values among the Einkorn wheat landrace populations.

Pop ID	1	2	3
1	****		
2	0.06	****	
3	0.06	0.12	****

Figure 6. The dendrogram representing the phylogenetic relationships among einkorn wheat landrace populations.

3.6.2. *Genetic diversity estimates in the einkorn wheat landraces sub-populations*

The seed samples were collected from the local farmers in the region of Bolu-Seben and Kastamonu-İhsangazi. Bolu-Seben-Güneyce, Bolu-Seben, and Kastamonu-İhsangazi populations were divided into 4, 5, and 4 sub-populations, respectively. The genetic diversity estimates at the locus level were performed for 13 sub-populations. The mean number of alleles, effective alleles, genetic diversity value, and polymorphic locus number were observed as 2.00, 1.39, 0.24, and 30.00 (100%), respectively (Table 10). The highest numbers of alleles, effective alleles, genetic diversity values, and polymorphic locus numbers were determined to be 1.33, 1.21, 0.13, and 10 (33%) in the sub-population of Bolu/Seben/Güneyce (21/1), respectively. Additionally, the highest effective allele number of 1.21 was observed in the Bolu/Seben/Güneyce (23/2) and Bolu/Yakuplar populations, too. The lowest number of alleles, effective allele, genetic diversity value, and polymorphic locus number were identified as 1.13, 1.09, 0.06, and 4 (13%) in the sub-population of Bolu/Haccağız /Beylik region, respectively (Table 10).

Table 10. The total genetic diversity estimates among einkorn wheat landrace sub-populations.

POP		Sub-pop	Sample Size	n_a	n_{ea}	H	# PL	% PL
1	Bolu/Seben/Güneyce Köyü (8/1)	1	3	1.17	1.11	0.06	5.00	0.17
	Bolu/Seben/Güneyce Köyü (21/1)	2	3	1.33	1.21	0.13	10.00	0.33
	Bolu/Seben/Güneyce Köyü (22/1)	3	2	1.23	1.17	0.10	7.00	0.23
	Bolu/Seben/Güneyce Köyü (23/2)	4	2	1.30	1.21	0.12	9.00	0.30
2	Bolu/Yakuplar Köyü	5	2	1.30	1.21	0.12	9.00	0.30
	Bolu/ Haccağız Köyü/Beylik Mevkii	6	2	1.13	1.09	0.06	4.00	0.13
	Bolu/Seben/Musasofular Köyü	7	2	1.20	1.14	0.08	6.00	0.20
	Bolu/Seben/Gerenözü Köyü	8	2	1.17	1.12	0.07	5.00	0.17
	Bolu/Seben/Nimetli Köyü	9	2	1.27	1.19	0.11	8.00	0.27
3	Siyez-Kastamonu/İhsangazi/Uzunoğlu Mah.	10	3	1.23	1.15	0.09	7.00	0.23
	Siyez-Kastamonu/İhsangazi/Çay Mah.	11	3	1.20	1.16	0.09	6.00	0.20
	Siyez-Kastamonu/İhsangazi	12	2	1.17	1.12	0.07	6.00	0.20
	Siyez-Kastamonu/İhsangazi/Koçcuağzı Köyü	13	2	1.17	1.12	0.07	5.00	0.17
		Mean	30	2.00	1.39	0.24	30.00	1.00

At the sub-population level, the total genetic diversity and the genetic diversity within the sub-populations were 0.25 and 0.09, respectively, while the genetic differentiation and gene flow between populations were 0.63 and 0.29, respectively (Table 11).

Table 11. The total genetic diversity and G (*F*) statistics estimates among einkorn wheat landrace sub-populations.

Sample Size	H_T	H_S	G_{ST}	N_m
30	0.25	0.09	0.63	0.29

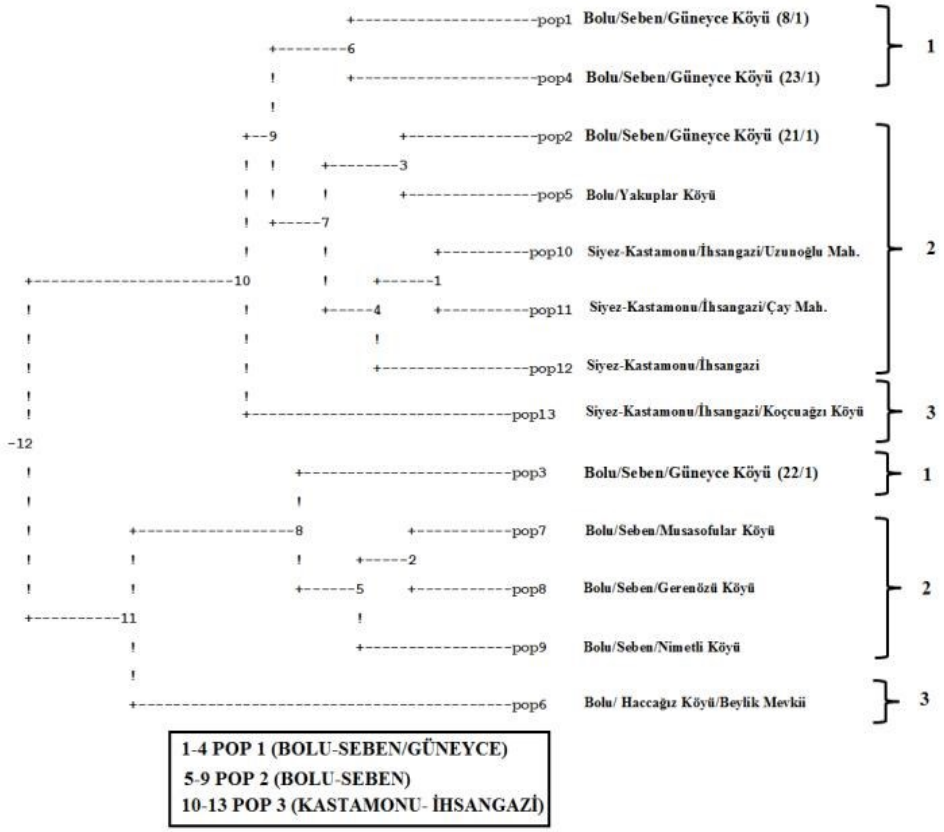
According to the genetic distance values, the highest genetic distance value was 0.49 between the sub-populations 4 and 6, while the lowest genetic distance value was 0.06 between the sub-populations 7 and 8, 10 and 11 (Table 12).

Table 12. The genetic distance values among the Einkorn wheat landrace sub-populations.

Pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1	****												
2	0.13	****											
3	0.29	0.12	****										
4	0.11	0.11	0.12	****									
5	0.15	0.08	0.22	0.15	****								
6	0.46	0.31	0.31	**0.49**	0.33	****							
7	0.27	0.17	0.14	0.24	0.21	0.18	****						
8	0.31	0.12	0.10	0.26	0.21	0.17	**0.06**	****					
9	0.33	0.17	0.14	0.27	0.21	0.22	0.11	0.07	****				
10	0.13	0.14	0.29	0.19	0.13	0.34	0.24	0.28	0.27	****			
11	0.08	0.09	0.23	0.15	0.09	0.37	0.21	0.25	0.24	**0.06**	****		
12	0.18	0.14	0.33	0.24	0.14	0.45	0.33	0.31	0.24	0.10	0.08	****	
13	0.14	0.17	0.35	0.19	0.17	0.42	0.31	0.33	0.33	0.16	0.15	0.10	****

A dendrogram constructed based on the genetic distance values, which were calculated according to iSSR data, indicated that the sub-populations were divided into sub-groups under two major groups. In general, in the first major group, Bolu-Seben/Güneyce and Kastamonu-İhsangazi populations were grouped together but in different subgroups, while in the second major group, Bolu-Seben populations were grouped. In the first major group, the population of Kastamonu-İhsangazi/Koçcuağzı was grouped as an outlier group, while in the second major group, the population of Bolu-Haccağız/Beylik region was grouped as an outlier group (Figure 7).

Figure 7. The dendrogram representing the phylogenetic relationships among einkorn wheat landraces and sub-populations.



4. DISCUSSION

According to the genetic diversity estimates, the average successful allele number and genetic diversity value were significantly high. One of the most important tools for determining genetic variation is the number of effective alleles. The number of successful alleles corresponded to the number of alleles contributing to genetic variation, roughly. The number of alleles included in the sample is very high, which shows that genetic variation is still very high. The extent of the genetic variation of self-pollinating plant species is very low, and the cross-pollination rate of the einkorn is less than 1 percent. However, the estimates of genetic variation observed in this study were quite high. One of the reasons was that einkorn wheat has been produced by local farmers for 10,000 years and passed down from generation to generation. Therefore, they harbor quite different gene/gene combinations in their gene pools, which may be the reason for the higher level of genetic diversity in their gene pools. According to the genetic diversity data observed at the locus level, Bolu populations had higher genetic diversity than Kastamonu populations. Considering the populations collected from both provinces and the number of samples analyzed, it made sense to have higher genetic diversity as the number of samples is higher in Bolu populations.

According to the genetic diversity data at the population level, the total genetic diversity was higher than the genetic diversity within the population. When the value of genetic differentiation among populations is > 15%, the genetic differentiation is substantially high. However, although the value of the genetic differentiation in this study appears to be high according to this criterion, it may not be that high compared to previous studies on einkorn wheat and other primitive landraces (Keskin *et al.*, 2015; Özbek *et al.*, 2011, 2012, 2013; Ozbek, 2021). The gene flow and genetic distance data also supported our study.

Three populations were divided into 13 sub-populations according to their gathering locations. In the sub-populations formed this way, the mean number of effective alleles and genetic diversity values were observed at very high levels. If the number of samples studied in sub-populations was large, estimates of genetic diversity would be observed at a higher level. While Bolu-Seben / Güneyce sub-populations showed higher values of genetic diversity values than other sub-populations, Kastamonu-İhsangazi sub-populations showed lower values of genetic diversity. The total genetic diversity values at the population level were quite high compared to the genetic diversity values at the locus level. On the other hand, the values of genetic diversity observed in subpopulations were quite low. Another remarkable result in sub-populations was that genetic differentiation was substantially high, whereas the level of gene flow was very low. Local farmers who produce local wheat exchange their seeds with neighboring farmers or farmers in other cities, buy local seeds from merchants, or mix them with their own seeds and plant them in their fields. This increases genetic diversity while eliminating the negative effects of seed depression. However, the high level of genetic differentiation observed in subpopulations in this study indicated that the villagers did not exchange their seeds or did so at very low levels. Villagers probably consume some of their harvested seeds while using some as seeds and sowing them in their fields for the next year. Since the same seeds are constantly used, genetic changes occur at different points in the genomes of the seeds in the hands of the villagers. The insufficient level of seed exchange and low cross-pollination levels due to selfing Three populations were divided into 13 sub-populations according to their gathering locations. In the sub-populations formed this way, the mean number of effective alleles and genetic diversity values were observed at very high levels. If the number of samples studied in sub-populations was large, estimates of genetic diversity would be observed at a higher level. While Bolu-Seben / Güneyce sub-populations showed higher values of genetic diversity values than those of other sub-populations, Kastamonu-İhsangazi sub-populations showed lower values of genetic diversity. The total genetic diversity values at the population level were quite high compared to the genetic diversity values at the locus level. On the other hand, the values of genetic diversity observed in subpopulations were quite low. Another remarkable result in sub-populations was that genetic differentiation was substantially high, whereas the level of gene flow was very low. Local farmers who produce local wheat exchange their seeds with neighboring farmers or farmers in other cities, buy local seeds from merchants, or mix them with their own seeds and plant them in their fields. This increases genetic diversity while eliminating the negative effects of seed depression. However, the high level of genetic differentiation observed in subpopulations in this study indicated that the villagers did not exchange their seeds or did so at very low levels. Villagers probably consume some of their harvested seeds while using some as seeds and sowing them in their fields for the next year. Since the same seeds are constantly used, genetic changes occur at different points in the genomes of the seeds in the hands of the villagers. The insufficient level of seed exchange and low cross-pollination levels due to selfing also cause low gene flow. As a result, genetic differentiation is increasing among the einkorn wheat populations and sub-populations. According to the genetic distance data, although, interestingly, the highest genetic distance is observed among Bolu sub-populations, the genetic distance data is consistent with other results in general. also cause low gene flow. As a result, genetic differentiation is increasing among the einkorn wheat populations and sub-populations. According to the genetic distance data, although, interestingly, the highest genetic distance is observed among Bolu sub-populations, the genetic distance data is consistent with other results in general.

The dendrogram constructed according to iSSR data for sub-populations is consistent with genetic distance and genetic differentiation values. When the analysis was done for only three populations, the Bolu and Kastamonu populations were grouped separately, while in the analyses of sub-populations, the Bolu-Seben/Güneyce sub-populations and the Kastamonu sub-

populations were clustered together but in different sub-groups in the first major group, while the Bolu-Seben sub-populations were clustered separately from the other groups. This is because einkorn wheat seeds have been distributed by Bolu Municipality to local farmers since 2014. Most likely, the einkorn wheat seeds distributed in the Bolu-Seben-Güneyce region originated from the einkorn wheat seeds grown in their fields. Apart from these, the eco-geographic (rainfall, humidity, temperature, daylight length, altitude, latitude, longitude, etc.) conditions in the regions where these populations were raised may have had a certain effect on genetic diversity and differentiation.

The genetic differentiation between the sub-populations was substantially higher (63%) than within populations (37%). The high level of genetic diversity determined in wheat landraces is related to some other functional factors. These factors might be, after the domestication process, domesticated wheat varieties that started to be cultivated by traditional farmers and the seeds have been sown for thousands of generations since then. The natural selection in the environment and farmers' interest in the wheat varieties they grew contributed to shaping the population structure. They also made selections on wheat they grow for their resistance to biotic and abiotic stress factors, and the amount of yield and yield stability in low-input agricultural systems (Ozbek, 2021; Zeven, 1999).

Inter-Retrotransposon Amplified Polymorphism (iRAP) markers indicated that Iranian diploid einkorn wheat (*Triticum monococcum* L. ssp. *monococcum*, *T. boeoticum* subsp. *boeoticum*, *T. boeoticum* subsp. *Thaoudar,* and *T. urartu*) had high genetic similarity due to high affinity and gene flow; however, *Triticum monococcum* L. ssp. *monococcum* was distinctively different from *T. boeoticum* and *T. urartu*, which were distant species to the other species studied (Eslami Farouji *et al*., 2015). Genetic diversity was investigated in 36 diploid wild einkorn wheats (*Triticum boeoticum*) by AFLP (Malaki *et al*., 2006), in diploid species belonging to the genus *Triticum* by RFLP (Le Corre & Bernard, 1995), and in 36 diploid wild einkorn wheats (*Triticum boeoticum*) from West Iran by RAPD, AFLP, and SSR markers (Naghavi *et al*., 2007). A larger collection of miRNAs and small RNA molecules was used for the analysis of *Triticum monococcum* L. ssp. *monococcum* plant samples grown under natural, drought, and salinity conditions. The appearance of 167 supposedly mature miRNA sequences belonging to 140 distinct miRNA families was suggested by next-generation technologies and bioinformatics analyses. In addition to a systematic study of scanned target genes within the *T. aestivum* L. genome, a comparative analysis was conducted to see target mirror genes that included the management of salt and drought (Ünlü *et al*., 2018). Ten cultivated einkorn (*Triticum monococcum* L. ssp. *monococcum*) landrace populations originating from Türkiye were investigated to determine the genetic diversity of high-molecular-weight (HMW) glutenin subunits and the gliadins. The cultivated einkorn populations in Türkiye displayed an enormous amount of genetic diversity for seed storage proteins of glutenin (H_e = 0.65) and gliadins (H_e = 0.17) (Keskin Şan *et al*., 2015; Ozbek, 2021).

5. CONCLUSION

The cultivated einkorn wheat [*Triticum monococcum* L. ssp. *monococcum* (2n = 2x = 14, A^mA^m)] and emmer wheat [*Triticum turgidum* ssp. *dicoccon* Schrank Thell. (2n = 4X = 28, *AABB*)] were the most popular crops until the early Bronze Age. Then, they started to be replaced by high-yielding and free threshing wheat varieties (*Triticum aestivum* L. 2n = 6X = 42, *AABBDD,* and *Triticum durum,* 2n = 4X = 28, *AABB*). Today, both species are relict and growing in Morocco, Tunisia, Italy, Spain, the Balkans, and Türkiye (Fritsch *et al*., 1996; Ozbek, 2021).

When reviewing the agro-morphological character averages, we found that one of the best characters was in population 27 of the IZA-Bolu/Göynük/Çaylak region for germination strength, root count, and root length. Germination power provides a better yield at harvest

because the population has better germination strength, the highest number of roots, and the best root length compared to other populations.

Population 21, IZA-Bolu/Seben/Güneyce Village, had the lowest mean coleoptile length, root count, root length, fresh root weight, and dry root weight.

The dendrograms presented show that some agro-morphological characteristics in certain populations do not correlate with those in other populations. For coleoptile length and root count, fresh root weight, and dry root weight, Population 21 had no relationship with the other populations. In population 27, the germination power and the root length did not correlate with each other. Population 15, on the other hand, did not correlate for fresh-root with the other populations, and Population 47 and Population 7 did not have a correlation for leaf weight with the other populations, and Population 1 did not correlate for germinating strength with the other population, and Population 35 did not correlate for coleoptile-length with the other populations.

Population 21 and Population 27 are the least related to the rest of the populations, which indicated that the characteristics of the other populations were different. While it has been found previously that the average Tukey HSD test also showed that Population 27 had the best characters, Population 21 had the worst characters.

Molecular markers have been used efficiently to represent phylogenetic relationships in plant species. An investigation of the nuclear and chloroplast genomes of diploid species using amplified fragment length polymorphism (AFLP) and simple sequence length polymorphism (SSLP) displayed that *T. urartu* was greatly differentiated from the other two *A-genome* species, and einkorn wheat had lower genetic diversity than that of *Aegilops* species (Mizumoto *et al*., 2002).

The wheat landraces have accumulated an enormous amount of genetic variation over thousands of years. The extent of variability, characterization, and partition of genetic diversity within a local germplasm collection are important criteria to determine the status of wheat landraces, particularly for future interests in their uses and for the improvement and efficient genetic diversity maintenance and utilization of plant species (Desheva *et al*., 2020).

Recently, people's interest in healthy foods has also increased interest in the cultivation of wheat landraces in Europe, and North African countries such as Morocco, Egypt, and Ethiopia, as well as in Türkiye. Nevertheless, it seems that einkorn wheat landraces are, today, growing only in Bolu, Çankırı, Çorum, Kayseri, Sinop, and Kastamonu provinces in Türkiye (Özberk *et al*., 2016).

The study found apparent differences between the populations in seven characters, and as can be noticed, there are differences between the populations in different cities in Türkiye. Accordingly, it is recommended that more extensive studies be carried out among the population on the most apparent characteristics through the germination of the population for a longer period until the crop ripens. In addition, they study the differences between the populations in terms of leaf length, PH, spike length, spike weight, number of seeds per spike, seed weight, and population study under different environmental conditions.

Also, Population 27 of IZA-Bolu/Göynük/ÇaylakKöyü is recommended to obtain the germination power of the crop and obtain productive power at harvest because the population has the highest germination power, the greatest number of roots, and the best root length compared to other populations.

Due to the presence of einkorn wheat in other countries, it is recommended to make a comparative study between the einkorn wheat in Türkiye and that in other countries for agro-morphological and molecular characteristics.

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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

Suliman Zommita: Conception and design, sampling, data collection and analysis, evaluation of results, writing/editing the manuscript. **Ozlem Ozbek:** Conception and design, data analysis, evaluation of results, editing the manuscript, supervising. **Gulgez Gokce Yildiz:** Data collection, editing the manuscript. **Omer Can Unuvar:** Data collection and editing the manuscript. **Ercan Selcuk Unlu:** Conception and design, data collection, evaluation of results, and editing the manuscript. **Nusret Zencirci:** Supervising conception and design, editing the manuscript.

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Research Article

Interactive effects of salicylic acid and jasmonic acid on secondary metabolite production in *Echinacea purpurea*

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Abstract: Secondary metabolites are highly beneficial to human health and have commercial and industrial values. So, this research aimed to study the effects of exogenous salicylic acid (SA) and jasmonic acid (JA) on some secondary metabolites in purple coneflower. A field experiment as a randomized complete block design with three replications was conducted in Shahrood, Iran. Treatments were the factorial arrangement of 3 SA (0, 0.5, and 1 millimole) and 4 JA concentrations (0, 5, 20, and 50 micromole). The non-linear regression procedure was employed to quantify the relation of these materials with each other. The results indicated that the SA effect on all ten measured secondary metabolites changed with changing the JA levels as there was the interaction between these elicitors. On average, most (7 out of 11) of the combined SA_JA levels up-regulated the production of secondary metabolites as compared to the plants not sprayed with SA and JA. In terms of average response to elicitation with 11 combined SA_JA levels, they ranked from higher to lower as the guaiacol peroxidase, hydrogen proxide (H_2O_2), polyphenol oxidase, glutathione S-transferase, superoxide dismutase, NADPH oxidase, total phenolic content, phenylalanine ammonia-lyase, anthocyanin, and flavonoid. A few secondary metabolites appeared to have a biphasic relationship with each other. For instance, over lower and medium values of NADPH oxidase activity, anthocyanin content increased linearly with increasing NADPH oxidase activity; over higher values of NADPH oxidase activity, it showed a plateau state.

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1. INTRODUCTION

Purple coneflower (*Echinacea purpurea) is a native plant to North America and* the most widely cultivated medicinal plant (*Kaiser et al., 2015*). Its commercial products have diversified to include capsules, tablets, powders, tinctures, teas, and other beverages, as well as personal care products (Patel *et al*., 2008). Many benefits have been reported for this plant, including chemoprevention, treating the toothache, bowel pain, snake bite, skin disorders, seizure, chronic arthritis, cancer, upper respiratory tract infections such as colds and flu, and as an immune stimulant (Patel *et al*., 2008). The *bioactive compounds of purple coneflower increase the number of white blood cells and spleen cells and improve the ability of phagocytosis by granulocytes (*Kaiser *et al.*, 2015). *The promotion of the immune system through the consumption of purple coneflower* products *has been attributed to alkamides, glycoproteins,*

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*polysaccharides, caffeic acid, and derivatives (*Kaiser *et al.*, 2015). The phenolic compounds, as one of the most important constituents of coneflower, contain very valuable organic materials.

The synthesis of above-mentioned bioactive compounds, known as secondary metabolites, requires expensive, difficult, and time-consuming procedures; in addition, the natural combinations of these compounds are more effective than artificially made ones. Therefore, different compounds including abiotic (e.g. titanium (IV) ascorbate), biotic (e.g. yeast extract), growth regulators (e.g. gibberellic acid), herbicides (e.g. glyphosate), and stress response molecules (e.g. methyl jasmonate; the volatile derivative of JA) have been employed to elicit the secondary metabolites in the different species of coneflower (Parsons *et al*., 2018).

SA and JA are known as elicitors and key signaling compounds in the induction process, leading to the accumulation of secondary metabolites. JA and SA respond to the biotic and abiotic stresses of the plants, and induce catalytic reactions by specific enzymes involved in the biosynthesis of phenolic compounds (Mendoza *et al*., 2018). SA regulates the phenylalanine ammonia-lyase (PAL) enzyme activity, which is a biosynthetic enzyme that catalyzes biosynthetic reactions related to the formation of defensive compounds such as superoxide dismutase and peroxidase (Torun *et al*., 2020).

Generally, JA tends to be involved in physiological and molecular responses. Physiological responses include activation of the antioxidant system superoxide anion radical, peroxidase, NADPH-oxidase, called antioxidant system (Karpets *et al*., 2014), accumulation of isoleucine, methionine, and soluble sugars (Wasternack, 2014), and regulation of stomatal closing and opening. JA has been shown to interact with plant hormones like ABA, GA, and IAA (Acharya & Assmann, 2009). Under the freezing and salt stress conditions, ABA and SA together positively regulate stress tolerance response (Horváth *et al*., 2015). Exogenous SA pretreatment significantly induced freezing tolerance of wheat via enhancing biosynthesis of ABA (Wang *et al*., 2018). In salt-stressed tomato plants, SA treatment has induced ABA biosynthesis and partially recovered lowered photosynthetic activity (Horváth *et al*., 2015). Pretreatment of barley plants with SA has led to a reduction in water deficit-resulted damage (Bandurska & Stroiński, 2005). SA and methyl jasmonate foliar application has increased the phenolic compounds in plant cell suspension cultures of *Thevetia peruviana* (Mendoza *et al*., 2018). Treated *Salvia miltiorrhiza* with SA in cell culture has had more PAL activity and contained more phenolic compounds (Dong *et al*., 2010). In an experiment on callus culture of *T. peruviana,* elicitation with the combination of 100 millimole of JA and 10 millimole of abscisic acid has resulted in an increased production of phenolic compounds (Rincón-Pérez *et al*., 2016). Methyl jasmonate and SA application have had a significant effect on antioxidant enzymes activity in many plants including *Arachis hypogaea* (Kumari *et al*., 2006).

Most of the studies regarding secondary metabolite elicitation of purple coneflower have been carried out within *in vitro* culture systems. Additional studies on the application of elicitors to field-grown purple coneflower would also be useful, since effects may differ considerably under the diversity of field conditions (Parsons *et al*., 2018). Recently, Mohebby *et al*. (2021) assessed the effect of SA and methyl jasmonate on three antioxidant enzymes (catalase, peroxidase, and superoxide dismutase), chicoric acid, and chlorogenic acid content of field-grown purple coneflower. The present field experiment aimed to study the effect of SA and JA on some other secondary metabolites of purple coneflower.

2. MATERIAL and METHODS

This field experiment was conducted at the Research Farm of Shahrood University of Technology, Shahrood (36° 25' N, 55° 01' E, and 1345 m asl), Iran in 2017. The mean annual value of temperature, precipitation, and relative humidity was 14 °C, 180 mm, and 48%,

respectively. Soil texture (0-30 cm depth) was loam-silt with 0.76% organic carbon, 15.54 ppm available phosphorous, 250 ppm available potassium, 0.06% total nitrogen, pH 7.2, and EC 1.5 dSm^{-1}.

The seeds of *Echinacea purpurea* were obtained from Pakan Seeds Company, Isfahan, Iran, and were sown in the nursery on 21 March. The transplants were planted on 5 June with a 30 cm separation between plants and 60 cm separation between rows. Each plot area was 9 m^2 (3 m × 3 m) with five rows. The experiment followed a randomized complete block design with three replications per treatment. Treatments were the factorial arrangement of 4 JA (0, 5, 20, and 50 micromole), and 3 SA concentrations (0, 0.5, and 1 millimole). At the onset of reproductive growth stage, JA and SA were sprayed on plants at a 10-day interval. The foliar application was repeated two more times at the mentioned interval. One week after the last spray, plant samples (the upper fully developed leaf) were taken to measure the following secondary metabolites.

H_2O_2 assay: The concentration of this material was determined following the Nelson method as described by Snell & Snell (1971). The reaction mixture consisted of 2.5 milliliter of 0.1% titanyl sulfate in 20% sulfuric acid (v/v), and 0.75 milliliter enzyme extract. The mixture was centrifuged at 5,000 g for 15 min at 25 ºC. The absorbance readings were taken at 410 nm to quantify the intensity of the yellow color that developed in the reaction. The extinction coefficient (ε_{410}) was 0.28 micromole $^{-1}$ cm^{-1}.

Superoxide dismutase (SOD) assay: Beauchamp and Fridovich (1971) method was adopted for SOD assay. In this method, riboflavin and methionine are used to produce superoxide in the presence of light. Superoxide causes the reduction of nitro blue tetrazolium and the formation of purple formazan. The reaction mixture was incubated under fluorescent light for 10 min and the absorbance was measured against a blank at 560 nm.

Guaiacol peroxidase (GPX) assay: The activity of GPX was measured by the method of Chance & Maehly (1955). A reaction mixture of 3 milliliter contained 0.1 mole phosphate buffer (pH 6.8), 30 millimole guaiacol, 30 millimole hydrogen peroxide, and 0.3 milliliter enzyme extract. Enzyme activity began by adding hydrogen peroxide to the reaction mixture. The increase in the absorbance of the reaction solution at 470 nm was recorded for one min.

NADPH oxidase assay: NADPH oxidase activity was determined according to Van-Gestelen *et al*. (1997) method. Briefly, nitro blue tetrazolium was converted to monoformazan by two $O_2^{\bullet-}$ molecules. This reduction was ascertained for one min at 530 nm. The reaction mixture (1 milliliter) contained 50 millimole Tris-HCl buffer (pH 7.8), 1 millimole $CaCl_2$, 0.1 millimole nitro blue tetrazolium, and 0.1 millimole NADPH. NADPH oxidase activity was expressed as micromole of nitro blue tetrazolium converted per minute per gram fresh weight of tissue.

Glutathione S-transferase (GST) assay: GST activity was determined as described by Gronwald and Plaisance (1998) which is the modified procedure of Habig *et al*. (1974, cited from Gronwald and hecked, 1998). The reaction medium contained 0.1 mole potassium phosphate (pH 6.5), 1 millimole glutathione, 1 millimole 1-chloro, 2,4-dinitrobenzene (CDNB), 1% absolute ethanol, and protein in a total volume of 1 milliliter. One unit of GST activity is defined as the formation of 1 micromole product min^{-1} at the temperature 25 °C.

Polyphenol oxidase (PPO) assay: PPO activity was determined by the method of Kar & Mishra (1976). The assay mixture (3 milliliter) contained 25 millimole phosphate buffer (pH 6.6), enzymatic extract (100 microliter), 0.1 millimole pyrogallol. The absorbance of the mixture was recorded at 420 nm. Enzyme activity began by adding pyrogallol to the reaction mixture.

Phenylalanine ammonia-lyase (PAL) assay: PAL activity was determined according to Wang *et al*. (2006), which is based on the cinnamic acid (CA) production rate. One milliliter of the

extraction buffer, 0.5 milliliter of 10 millimole L-phenylalanine, 0.4 milliliter of double-distilled water, and 0.1 milliliter of enzyme extract were incubated at 37 ^{o}C (a temperature at which the peak activity of PAL takes place) for 1 hour. The reaction was terminated by adding 0.5 milliliter of 6 mole HCl; then the product was extracted with 15 milliliter ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 milliliter of 0.05 mole sodium hydroxide, and the CA concentration was quantified based on the absorbance at 280 nm.

Total phenolic content (TPC) assay*:* TPC was determined using Folin–Ciocalteu's reagent (Singleton & Rossi, 1965). Briefly, 0.5 milliliter of the diluted extract was reacted with 2.5 milliliter of 0.2 mol per liter Folin Cicoalteu reagent. After 4 min, 2 milliliter of saturated sodium carbonate (about 75 g L^{-1}) was added and then, the solution was incubated for 120 min at room temperature. The absorbance readings of the resulting blue-colored solution were taken at 760 nm. A standard curve, prepared with 100, 200, 300, 400, and 500 mg L^{-1} of gallic acid, was adopted as a reference standard and TPC was expressed as gallic acid equivalent (mg GAE)g^{-1} fresh weight of the sample.

Anthocyanin assay: Anthocyanin content was determined as described by Mita *et al*. (1997). Fresh leaf sample (20 mg) was homogenized with 3 milliliter of 1% (v/v) hydrochloric acid in methanol and then the extraction was kept under dark conditions at 4 °C for 24 h. The mixture was centrifuged at 10,000 g for 15 min and then the absorbance of the supernatant was recorded at 530 and 657 nm. One unit of anthocyanin equals one unit of absorbance [A530 - (0.25 × A657)] per milliliter of extraction solution.

Flavonoid assay: The aluminum chloride colorimetric method was applied to estimate the flavonoid content as described by Chang *et al*. (2002). Leaf sample extract (0.5 milliliter of 1:10 g $milliliter^{-1}$) in methanol was mixed with 1.5 milliliter of methanol, 0.1 milliliter of 10% aluminum chloride, 0.1 milliliter of 1 mole potassium acetate, and 2.8 milliliter of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was recorded at 415 nm. Quercetin solutions (12.5 to 100 g $milliliter^{-1}$ in methanol) were utilized to make the calibration curve.

Statistical analysis: The statistical analysis, including analysis of variance and mean comparison based on the least significant difference (LSD) method, was performed, using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). The following equations were applied to express the biphasic relationship between secondary metabolites, using NLIN procedure of SAS software:

$$\begin{aligned} Y &= a + b \times X && \text{If } X < X_0 \\ Y &= a + b \times X_0 && \text{If } X \geq X_0 \end{aligned} \quad (1)$$

$$\begin{aligned} Y &= a + b \times X_0 && \text{If } X \leq X_0 \\ Y &= a + b \times X && \text{If } X > X_0 \end{aligned} \quad (2)$$

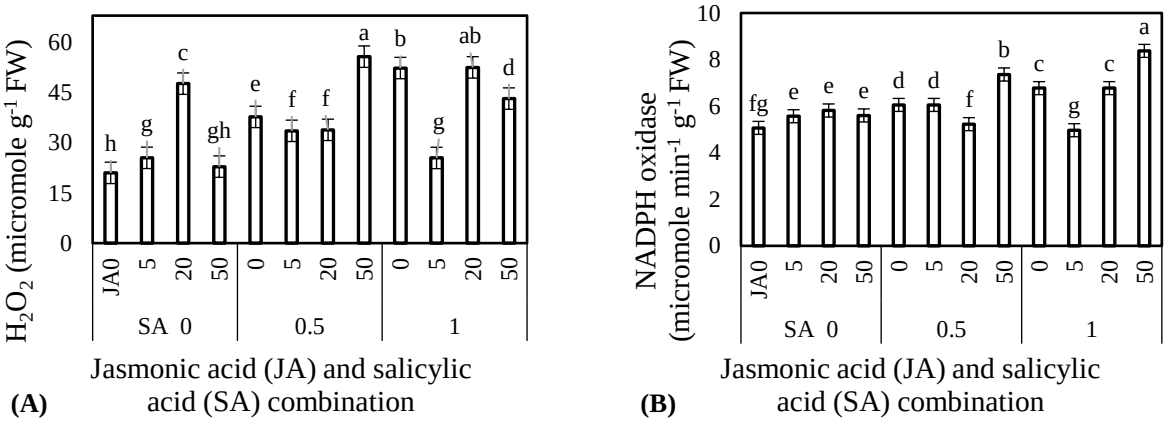
Where Y and X are secondary metabolites, a intercept, b slope, and X_0 the value of X at which the relation of Y with X shows a remarkable change.

3. RESULTS

The results of analysis of variance indicated that both simple and interactive effects of SA and JA were significant (p <0.01) on all measured secondary metabolites (data not presented). The mean values in Figure 1, pertaining to the H_2O_2 content of purple coneflower in response to varying levels of SA and JA, revealed considerable differences with each other. Under no SA (SA0) and 1 millimole SA application conditions (SA1), the higher H_2O_2 content was obtained with application of 20 millimole JA as compared to the other JA levels; while under 0.5

millimole SA spraying conditions, the higher H_2O_2 content was found upon elicitation with 50 millimole JA.

Figure 1. Interactive effects of JA (micromole) and SA (millimole) on H_2O_2 content (A), and NADPH oxidase activity (B) in purple coneflower.



In terms of NADPH oxidase activity in plants that were not sprayed with SA (SA0), treatments 5, 20, and 50 micromole JA behaved equally with non-significant differences among each other; these treatments were statistically superior to control (JA0) (Figure 1). The highest and second highest NADPH oxidase activity was found upon elicitation with 1 millimole SA and 50 micromole JA, and with 0.5 millimole SA and 50 micromole JA, respectively. As it is shown in Figure 2, the biphasic relationship was obtained between H_2O_2 content and NADPH oxidase activity; with increasing NADPH oxidase activity from 4.8 to 6.338 micromole min^{-1} g^{-1} leaf fresh weight, the H_2O_2 content was surged with a sharp slope (Table 1); with more increase in NADPH oxidase activity, it showed a plateau state.

Figure 2. Biphasic relation of H_2O_2 content with NADPH oxidase activity (A), and of anthocyanin content with NADPH oxidase activity (B).

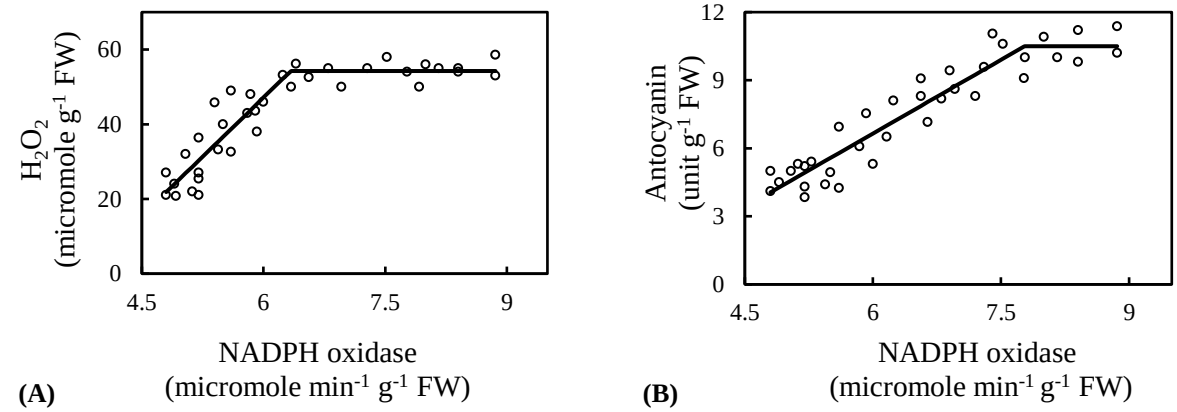


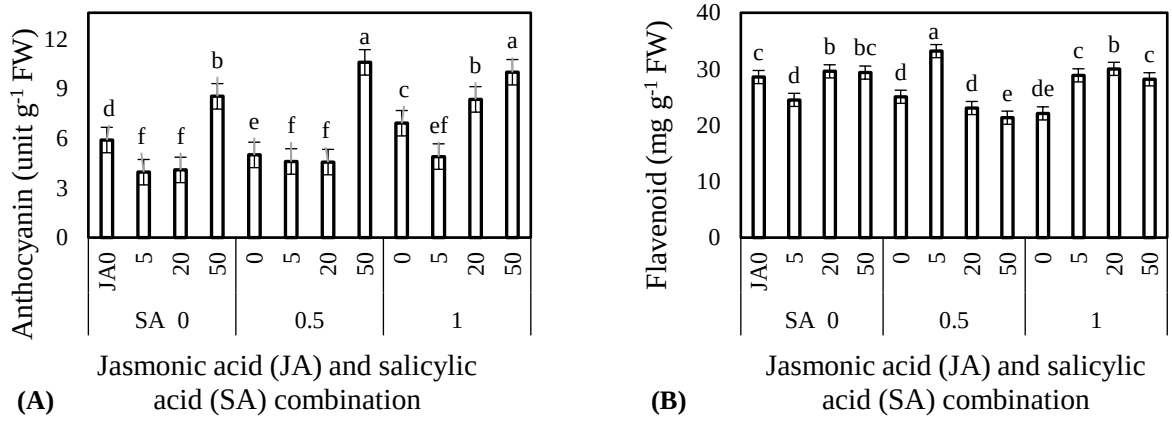
Table 1. The parameters ± standard error of non-linear-regression-based relationship between some secondary metabolites.

[a] Secondary metabolite	Intercept (a)	Slope (b)	X_0
H_2O_2 - NADPH oxidase	-79.224 ± 13.68	21.059 ± 2.52	6.338 ± 0.14
Anthocyanin - NADPH oxidase	-6.332 ± 1.07	2.164 ± 0.17	7.778 ± 0.22
Anthocyanin - GST	15.059 ± 1.21	-0.587 ± 0.09	17.924 ± 0.82
GPX - NADPH oxidase	-27.567 ± 5.12	7.117± 0.89	6.840 ± 0.18

[a] GST: Glutathione S-transferase; GPX: guaiacol peroxidase.

Regarding anthocyanin content, 50 millimole JA was superior to the other three JA levels; this was true for plants sprayed with all levels of SA (Figure 3). Surprisingly, for plants treated with all SA levels, application of 5 micromole JA decreased anthocyanin content to a level lower than control. As it is shown in Figure 2, over lower and medium values of NADPH oxidase activity, anthocyanin content increased linearly with increasing NADPH oxidase activity; over higher values of NADPH oxidase, it remained constant. The effect of JA on flavonoid content was considerably changed with changing SA levels (Figure 3). The highest and lowest flavonoid content was obtained for plants treated with 0.5 millimole SA and 5 micromole JA, and with 0.5 millimole SA and 50 micromole JA, respectively.

Figure 3. Interactive effects of JA (micromole) and SA (millimole) on anthocyanin (A), and flavonoid contents (B) in purple coneflower.



The treatment combination of 1 millimole SA and 5 micromole JA, and of 1 millimole SA and 20 micromole JA behaved equally with no significant difference between each other, and the highest SOD activity of 34.5 and 35.33 micromole g^{-1} FW were recorded in these treatment combinations, respectively (Figure 4). Averaged over JA levels, the plants elicited with 1 millimole SA consisted of lower amounts of GST activity than those elicited with no SA and 0.5 millimole SA (Figure 4). Under no SA application conditions, the value of GST activity increased linearly with increasing GA levels from 0 to 20 micromole. The treatment combination of no SA application and 20 micromole JA application got the highest position with 28.07 micromole g^{-1} FW GST activity. Over lower values of GST activity, there was an inverse relation between anthocyanin content and GST activity; over higher values of GST activity, anthocyanin content remained constant (Figure 5).

Figure 4. Interactive effects of JA (micromole) and SA (millimole) on SOD activity (A), and GST activity (B) in purple coneflower.

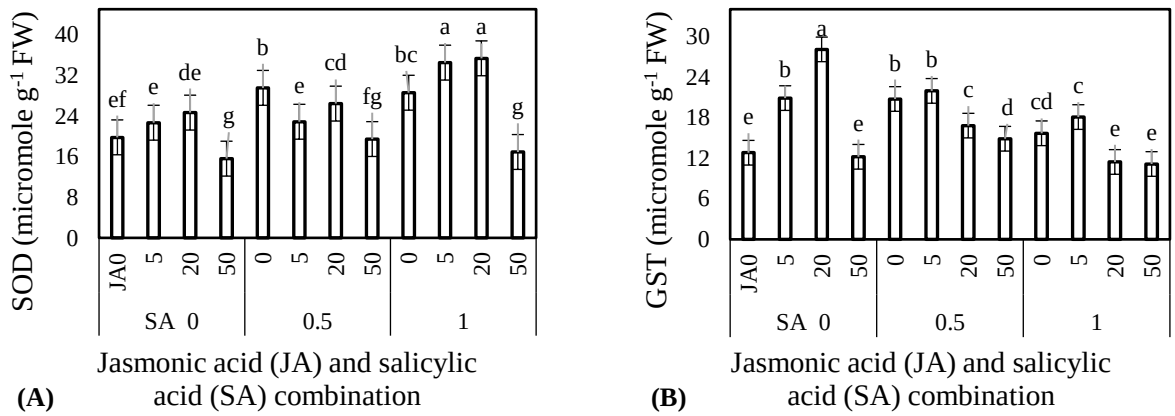


Figure 5. Biphasic relation of anthocyanin content with GST activity (A) and of GPX activity with NADPH oxidase activity (B).

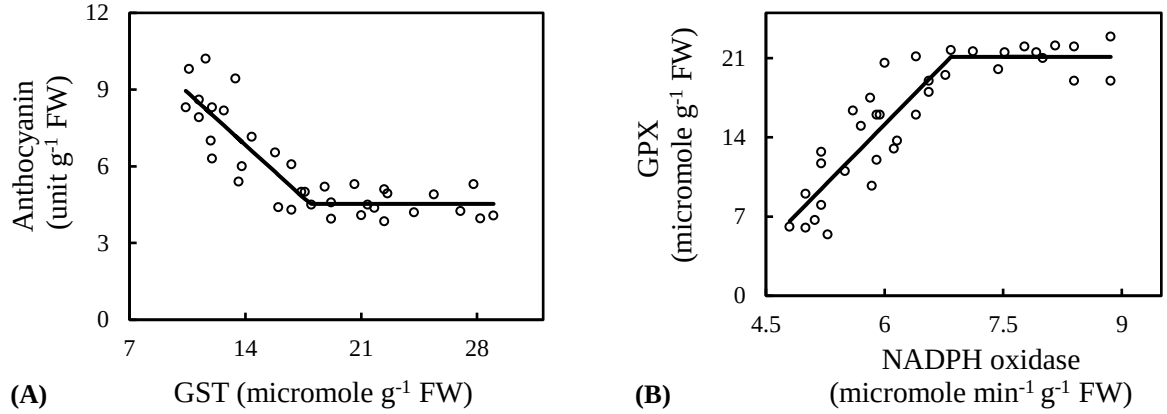
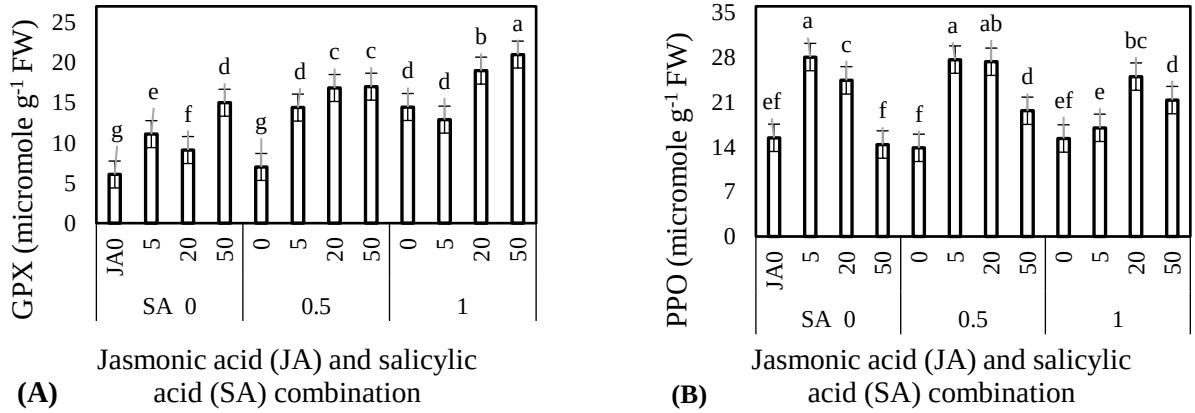
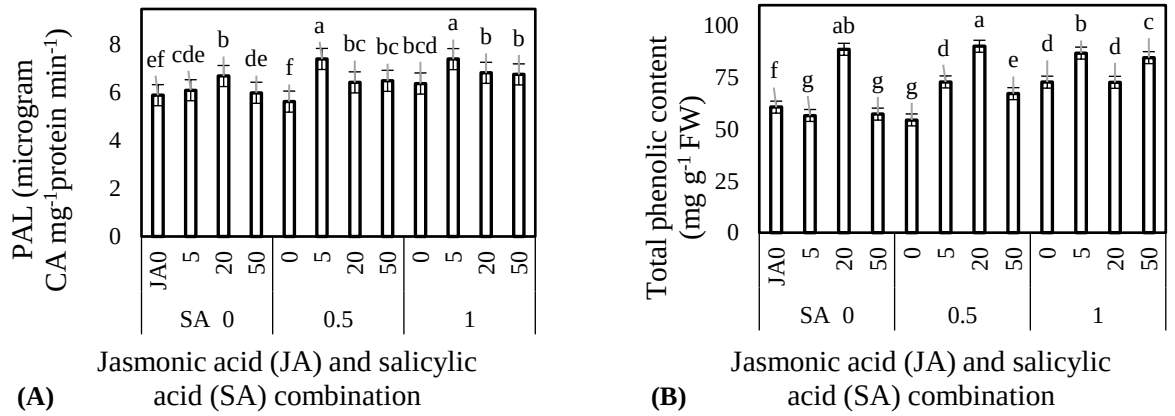


Figure 6. Interactive effects of JA (micromole) and SA (millimole) on GPX activity (A), and PPO activity (B) in purple coneflower.



All combined levels of SA_JA, except 0.5 millimole SA and no JA application, positively influenced GPX activity as compared to control (Figure 6). The highest and second highest GPX activity was obtained upon elicitation with 1 millimole SA and 50 micromole JA, and with 1 millimole SA and 20 micromole JA, respectively. Over lower values of NADPH oxidase activity, a positive relationship was found between GPX and NADPH oxidase activities; over higher values of NADPH oxidase activity, GPX activity showed a plateau state (Figure 5). In the context of PPO activity, there was no significant difference between the treatment combination of 0 millimole SA_5 micromole JA, 0.5 millimole SA_5 micromole JA, and 0.5 millimole SA_20 micromole JA; these three treatment combinations attained the greatest PPO activity (Figure 6).

Figure 7. Interactive effects of JA (micromole) and SA (millimole) on phenylalanine ammonia-lyase (PAL; CA: cinnamic acid) activity (A), and total phenolic content (B) of purple coneflower.



In terms of PAL activity, 5 micromole JA application attained superiority when combined with 0.5 and 1 millimole SA (Figure 7). The lowest PAL activity (5.63 microgram CA mg^{-1} protein min^{-1}) was obtained upon elicitation with no JA and 05 millimole SA. Under no SA application conditions, only one JA level (20 micromole) up-regulated TPC as compared to control (Figure 7); while under 0.5 and 1 millimole SA application conditions, more JA levels promoted TPC to a level higher than control.

4. DISCUSSION and CONCLUSION

The results indicated that elicitation with SA and JA induced oxidative stress as H_2O_2 content was soared (Figure 1). This induction was highly intensive (2.6-fold enhance as compared to control) upon elicitation with 0.5 millimole SA and 50 micromole JA. The change in reactive oxygen species (ROS) generation, including H_2O_2, is attributable to NADPH oxidase involvement, as Rouet *et al*. (2006) reported that the oxidative response in tobacco, induced by hypo-osmolarity, is originated from NADPH oxidase activity. In our experiment, although NADPH oxidase activity was intensified by most of SA and JA levels (Figure 1), part of the H_2O_2 may not have been originated from NADPH oxidase activity since with increasing NADPH oxidase activity from 6.338 to 8.86 micromole min^{-1} g^{-1} FW, H_2O_2 content remained constant (Figure 2). The cell wall-bound peroxidase (Bolwell *et al*., 2002), oxalate oxidase (Hu *et al*., 2003), amine oxidase (Angelini & Federico, 1989), and quinone reductase (Schopfer *et al*., 2008) might be involved in H_2O_2 generation.

Anthocyanin has nutritional value and a healing effect on cardiovascular and neurodegenerative diseases in humans (Mattioli *et al*., 2020). Its content tended to be increased considerably in response to the high level of (50 micromole) JA application (Figure 3). Five out of 11 combined levels of SA_JA promoted anthocyanin accumulation as compared to control. The accumulation of anthocyanin is triggered by biotic and abiotic stresses; ROS scavenging is one of the anthocyanin's important roles (Agati *et al*., 2020). In many other plants, including radish (Sakamoto & Suzuki, 2019), the elicitors like methyl jasmonate have also caused an increase in the accumulation of anthocyanin. It seems that anthocyanin content is not up-regulated by higher values of NADPH oxidase activity; since, as it was shown in Figure 2, over lower and medium values of NADPH oxidase activity, anthocyanin content enhanced linearly with increasing NADPH oxidase activity; over higher values of NADPH oxidase activity, it showed a plateau state.

Four out of 11 combined levels of SA_JA enhanced the flavonoids content to a level higher than control (Figure 3). The maximum increase (16%) was obtained upon elicitation with 0.5 millimole SA and 5 micromole JA. A review report by Agati *et al*. (2020) supports a remarkable

antioxidant function served by flavonoids in plants exposed to a wide range of stressors-resulted oxidative stress. Eight combinations of SA_JA levels intensified GST activity (Figure 4). These results are in tune with other reports regarding the up-regulation of GST activity by SA and even exogenous H_2O_2 application in other plants (Gong *et al*., 2005). Over lower values of GST activity, there was an inverse relation between anthocyanin content and GST activity; over higher values of GST, no relation was found between them (Figure 5). Such a relationship between these two attributes in the leaf might be due to GST-mediated transportation of anthocyanin from the leaf and accumulation in other organs like stem and/or root. GST involves diverse aspects of plant physiology, including signal transduction, regulatory functions, and transport of anthocyanin from the cytosol to the vacuole for storage (Estévez & Hernández, 2020).

GPX, a heme-containing enzyme, can effectively quench the reactive intermediary forms of O_2 and peroxy radicals under stressed conditions (Sharma *et al*., 2012). Ten combinations of SA_JA levels, especially 1 millimole SA and 50 micromole JA (more than 200% increase as compared to control), resulted in the promotion of GPX activity (Figure 6). The intensification of GPX activity upon exogenous application of SA has also been evidenced in the cell suspension culture of *Scrophularia kakudensis* (Manivannan *et al*., 2016). The SOD activity is up-regulated in response to elicitation-resulted enhance in ROS formation (Kolupaeva & Yastreb, 2021). This antioxidant enzyme has a critical role in defense mechanisms by converting $O^{\cdot-}_2$ to H_2O_2 in cytosols, chloroplasts, and mitochondria. In the present study, elicitation with five combinations of SA_JA levels intensified the SOD activity (Figure 4). The effect of 5 and 20 micromole JA on SOD activity was the highest only when combined with a high level (1 millimole) of SA. This is an interaction between SA and JA, which implies the co-potentiated production of ROS by these elicitors. Our results are in agreement with the findings that have been reported previously (Mohebby *et al*., 2021).

Many reports show a positive correlation between PPO expression and resistance/tolerance to biotic stresses (Taranto *et al*., 2017). The present study is the first report that deals with the effect of JA and SA elicitors on the activity of PPO in purple coneflower. PPO activity appeared to be up-regulated upon elicitation of plant with seven combinations of SA_JA levels (Figure 6). Therefore, the exogenous application of JA and SA mimics the response of a wound signal or a pathogen attack, which triggers a defense reaction in plants by inducing the oxidative burst. In plants, the deamination of L-phenyl alanine to produce trans-cinnamic acid and ammonia is known as the first step for phenylpropanoid skeleton biosynthesis (Bagal *et al*., 2012). This reaction is catalyzed by the PAL enzyme and is often regarded as a key step in the biosynthesis of the phenylpropanoid compounds. Eight combinations of SA_JA levels resulted in an enhanced PAL activity (Figure 7). Our results are in accordance with the previous studies regarding the impact of SA and methyl jasmonate on wheat infected by *Pratylenchus thornei* (Ketabchi *et al*., 2015).

Phenolic compounds are highly valuable as they include biochanin, homogenistic acid, and 12 more beneficial materials (Ho *et al*., 2020). In comparison with control, eight combinations of SA_JA levels, especially 0.5 millimole SA_20 micromole JA (1.47 folds of control), caused up-regulation of TPC (Figure 7). In previous studies on purple coneflower, the methyl jasmonate and SA have also increased two derivatives of phenolic compounds, including chlorogenic acid (Mohebby *et al*., 2021). Generally speaking, in the present study JA and SA elicitation activated the defense responses of purple coneflower, thereby up-regulated the production of all measured secondary metabolites which have commercial and industrial values. Given the fact that a diet rich in secondary metabolites would be beneficial to human health, the elicitation of these metabolites in all edible plants including vegetables should be a major field of research in the future.

As a conclusion, on average, 7 out of 11 combinations of SA_JA levels increased the content/activity of measured secondary metabolites as compared to control. In terms of average response to elicitation with 11 combined levels of JA and SA, the secondary metabolites ranked from higher to lower as the GPX (2.37-fold of control), H_2O_2 (1.86), PPO (1.38), GST (1.36), SOD (1.27), NADPH oxidase (1.23), TPC (1.2), PAL (1.11), anthocyanin (1.1), and flavonoid (0.94). These metabolites have direct and indirect relations with each other. Here, segmented regression could quantify the relation of a few ones which had the simple biphasic relationship (Figures 3 and 5). In future studies, the artificial neural network procedure must be employed to quantify the relation of all these metabolites together. This procedure has successfully been used to quantify complicated relationships in many other fields of research (e.g. Gholipoor *et al*., 2012; 2013; 2019; Salehzadeh *et al*., 2016).

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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 Fatemeh Rasoli and Manoochehr Gholipoor.

Authorship Contribution Statement

Authors are equally participated in conducting the experiment and analyzing the results.

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Susceptibility of bacterial species isolated from mares to ozonated sunflower oil

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Abstract: Sunflower oil is known for its therapeutic properties and culinary use. It is an important alimentary source of tocopherol and unsaturated fatty acids, and is used especially for wound healing. Studies on its antimicrobial potential, however, are lacking. The ozonation of oils of vegetable sources has been explored to enhance their therapeutic properties; however, studies that provide evidence of such benefits are still lacking. In the field of veterinary medicine, such data are even more scarce. In this study, the antimicrobial activity of ozonated sunflower oil was compared to that of non-ozonated oil, in an in vitro system, against strains of *Staphylococcus aureus* and *Escherichia coli*, isolated from intrauterine lavages of mares with endometritis. Tests were conducted using the minimum inhibitory concentration method. The ozonated oil was effective against *S. aureus*, whereas it was not against *E. coli* isolates. Our data open doors for discussion on the use of sunflower oil, with or without ozone treatment, for therapeutic purposes in veterinary medicine.

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Sunflower oil, antimicrobial, *Escherichia coli*, *Staphylococcus aureus.*

1. INTRODUCTION

The world is facing a growing scenario of bacterial, fungal, and viral resistance to the currently available antimicrobial drugs, and the pharmaceutical industries do not show interest in developing new synthetic or semi-synthetic active molecules (Lai *et al*., 2022; Weis *et al*., 2022). This situation is even more complex in the field of Veterinary Medicine, as there are few studies that provide solid evidence in this context, but high levels of resistance are expected (Schwars *et al*., 2017).

In this context, exploring natural products as antimicrobials is more important than it sounds, as they have the potential to open doors for more effective and less expensive treatments, especially regarding infectious diseases (Dias-Souza *et al*., 2017). Studies with natural products usually do not require immediate technology transfer or very advanced technologies (although they are widely necessary in further steps of the research). The complex structure of

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phytomolecules, the availability of the plants in the environment, and the possibility of cultivation in controlled conditions make research in this field even more compelling (Dias-Souza *et al*., 2018).

Sunflower (*Helianthus annus* L.) oil, obtained from the seeds (one of the main oil crops in the world), is widely used for culinary and food technology purposes, which include cooking by frying, food packaging, and preservation (Kassab *et al*., 2019; Filho & Egea, 2021). The byproducts of the oil, often associated to negative environmental impacts and considered unuseful, are now relevant in the current global trend of implementation of greener industrial processes (Rai *et al*., 2016; Rauf *et al*., 2017). Concerning its biological properties, sunflower oil is mostly known by its wound healing and anti-inflammatory potentials, which are associated to its unsaturated fatty acids (Poljšak *et al*., 2020). Its antimicrobial activity, however, remains poorly described.

Here we investigated the antimicrobial potential of ozonated and conventional (non-ozonated) sunflower oil. There is a growing interest in the use of ozonated oils for the treatment of infectious and/or inflammatory diseases, and some commercial formulations are already available in the pharmaceutical market for human and veterinary use. Ozone is largely known for its biological properties such as wound healing and antimicrobial activity (Di Mauro *et al*., 2019); however, the efficacy and safety of ozone-treated oils remain in discussion. Ozone reacts in different ways with the molecules present at the oils, and products such as peroxides can cause DNA and cell membrane damage in determined conditions (Krkl *et al*., 2016).

In this study we show that conventional and ozonated sunflower oil are effective against *Staphylococcus aureus* and *Escherichia coli* strains isolated from mares with endometritis, a common condition that prevents mares of having a successful pregnancy. These bacterial species are among the most prevalent in the altered intrauterine microbiota of mares with the disease (Ávila *et al*., 2022). Our data open doors for more studies exploring sunflower oil to treat endometritis in mares.

2. MATERIAL and METHODS

2.1. Microorganisms

Ten clinical isolates of *S. aureus* and of *E. coli* from mares with endometritis were selected from the bacterial collection of the research laboratory from Pitágoras College (MG, Brazil). The isolates were cultivated in sterile BHI broth (35±2 °C, 18 h) prior to the experiments.

2.2. Preparation of the Oils

The oils with and without ozone treatment were purchased from local compounding pharmacies. For the antimicrobial activity assays, the oils were prepared in 0.9% sterile saline solution with 0.5% Tween 80 to increase their miscibility in water. A stock solution (4.1 mg/mL) was prepared on the same day of the experiments.

2.3. Antimicrobial Assays

Minimal inhibitory concentration (MIC) of the oils was determined in triplicate following CLSI standards and a protocol standardized by our group using 96-wells polystyrene plates (Dias-Souza *et al*., 2017; CLSI, 2018), with slight modifications. The stock solution of the oils was diluted in sterile 0.9% saline to reach final concentrations in the 96-wells plates ranging from 1024 to 8 µg/mL (100 µL). The overnight-grown bacterial inoculum was diluted in sterile Mueller-Hinton broth (Himedia) to reach the final concentration of $5x10^4$ CFU/mL (100 µL). The plates were incubated overnight at 35±2 °C. Next, we performed resazurine staining (0.1 g/L, 20 µL). The lowest concentration in which no color change from blue to pink was observed in the plates was considered the MIC. We used a 0.9% sterile saline solution with 0.5% Tween 80 as a negative control.

3. RESULTS and DISCUSSION

The ozonated sunflower oil was active against *S. aureus*, whereas the conventional oil was not active to both species even at the highest concentration tested. The ozonated oil was also not active against *E. coli* strains as well (Table 1). Negative control was also not active, as expected.

Table 1. Susceptibility of the isolates to ozonated and conventional sunflower oil

Bacterial species	MIC (μg/mL)	
	Ozonated sunflower oil	Conventional sunflower oil
E. coli	NA	NA
S. aureus	512	NA

Data is referent to all strains. NA: not active.

One may question why we did not explore higher concentrations of the oil in this study. MIC tests are conducted from values ranging from 1024 to 8 μg/mL as to simulate safe *in vivo* concentrations for treating infectious diseases (CLSI, 2018). Substances of elevated MIC values are not of interest for eventual clinical use, as large (and potentially toxic) amounts of them would be necessary (Karasawa & Steyger, 2011).

E. coli is a Gram-negative bacterial species naturally present at the intestinal tract of mammals. It can also behave a pathogen associated to several diseases such as urinary tract infections, intestinal infections, and, in mares and other animals, endometritis (Ávila *et al*., 2012). *S. aureus* is a Gram-positive bacterial species that is commensal to humans and animals, being associated to several skin and internal soft tissues infectious diseases. Recent studies suggest that both *E. coli* and *S. aureus* are among the most frequent bacterial species isolated from endometritis in mares. In the present study, the conventional oil was not effective against any of the species. However, the ozonated oil was effective against *S. aureus*, a possible effect from the peroxides generated by the ozonation process (Krkl *et al*., 2016; Di Mauro *et al*., 2019). Interestingly, the ozonated oil was also not effective against *E. coli.*

Studies that adequately investigated the antimicrobial potential of sunflower oil remain scarce. Although sunflower oil is not clearly associated to any cytotoxic events, previous studies indicate that concentrations in mg/mL scale would present considerable antimicrobial activity. A commercial formulation of ozonated sunflower oil was active against ATCC strains of *S. aureus* and *E. coli* at 9.5 and 4.75 mg/mL, respectively (Sechi *et al*., 2001). Curiously, a study described that both ozonated and conventional sunflower oils were active against ATCC strains of *S. aureus* and *E. coli*, with MIC values of 4.5 and 9.5 mg/mL, respectively (Diaz *et al*., 2006). Our data is partially in agreement with that of these studies, considering the effectiveness of the oil on *S. aureus* isolates.

Interestingly, an investigation suggested that fungal and yeast species associated to dermatophytosis can be more susceptible to sunflower oil than *E. coli* (Tabassum and Vidyasagar, 2014). A recent *in vivo* study described that a commercial ozonated sunflower oil reduced the bacterial counts of mares with endometritis, including *E. coli*, using 50 mL of the formulation *in uterus* (Ávila *et al*., 2022). Full eradication was not achieved, and high concentrations were necessary to reduce bacterial counts.

4. CONCLUSION

The ozonated sunflower oil was active against *S. aureus* isolated from mares with endometritis, but was not effective against *E. coli*, suggesting that treatments with this product may not be effective. The treatment of infectious diseases is mostly empiric in clinical routines, and the use of substances of known effectiveness is of paramount relevance to prevent bacterial resistance.

More studies are necessary to confirm the safety of the oil in higher concentrations, in which they might be effective.

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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

Gabriel Souza dos Santos: Conducted the experiments, drafted the manuscript. **Arthur Azevedo Perpétuo**: Conducted the experiments, drafted the manuscript. **Marcus Vinícius Dias-Souza**: Designed the study, supervised experiments, wrote the final version of the manuscript.

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Research Article

Oxidative stress induced by fluorine in *Xanthoria parietina* (L.) Th. Fr.

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Abstract: In our work we were interested in the toxicity of fluorine on the various parameters of stress: chlorophyll, proteins, and antioxidant system in the lichen *Xanthoria parietina* (L.) Th. Fr., and for this purpose, lichen thalli were treated by sodium fluoride (NaF) at concentrations of 0, 0.5, 1.0, 5.0 and 10.0 mM, for time scale 0, 24, 48 and 96 h. The analysis results obtained revealed that all the parameters evaluated showed significant variations compared to those of the controls. From the analysis results obtained, it was noted that chlorophyll a (C_a), chlorophyll b (C_b) and total chlorophyll (C_{a+b}) decreased correlating with exposure times to NaF (r = -0.785, $p < 0.001$; r = -0.955, $p < 0.001$; r = -0.899, $p < 0.001$, respectively), with a significant increase of $C_{a/b}$ ratio ($p = 0.00572^{**}$) showing that C_b was more affected than C_a. However, hydrogen peroxide (H_2O_2) increased (r = 0.949, $p < 0.001$). In correlation with NaF concentrations, Glutathione (GSH) increased (r = 0.969, $p < 0.001$), while proteins decreased (r = -0.872, $p < 0.001$). Furthermore, results showed that catalase activity (CAT) increased correlating with increasing exposure time of *X. parietina* to increasing concentrations of NaF. Long-term exposure (48 h -96 h) caused a significant decrease in GSH content ($p = 0.02^{*}$) followed by total destruction at time 96 h.

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Chlorophyll,
Proteins,
Antioxidant system,
Lichen.

1. INTRODUCTION

In their habitat, lichens are exposed to severe abiotic stresses such as desiccation and temperature extremes (Beckett *et al*., 2021), salinity (Chowaniec & Rola, 2022), heat (Kraft *et al*., 2022) heavy metals (Rola, 2020), and fluoride (Roberts & Thompson, 2011).

Plants endured significant biochemical and physiological changes as a result of the stressful environment. Chlorophyll degradation is the most common metric used to assess the toxicity of air pollution on lichens (Sujetovienė, 2015).

Fluoride is found in a variety of environmental matrices and even at low quantities and is one of the most phytotoxic chemical elements for plants (Banerjee & Roychoudhury, 2019). It affects the metabolic activity of plants by decreasing nutrient uptake, germination,

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photosynthesis, growth, and productivity (Sharma & Kaur, 2018). Fluoride toxicity has also a negative impact on enzyme activity, protein synthesis, gene expression patterns, and the formation of reactive oxygen species (ROS) (Choudhary *et al*., 2019).

The most important adaptation mechanism used by lichens for tolerance to stressful conditions is the scavenging of ROS. To prevent ROS-induced damage, plants synthesize many enzymatic components like catalase (Lei *et al*., 2022) and non-enzymatic components like glutathione (Hasanuzzaman *et al*., 2020), and change their protein composition (Amnan *et al*., 2022). In response to abiotic stress, plants also produce H_2O_2 as one of the ROS (Zhang, 2022). Under stressful conditions, GSH levels rise in plants (Nahar *et al*., 2017), and it is one of the plant's adaptive methods for combating and tolerating stress (Gong *et al*., 2018). Catalase activity also increases in plant under fluoride stress correlating with high concentration (Sharma & Kaur, 2019) and with exposure time (Sharma *et al*., 2019).

The study's aim is to explore the harmful effect of fluorine which is in the form of sodium fluoride (NaF) on the lichen *Xanthoria parietina*, by measuring contents of chlorophyll, proteins, catalase, hydrogen peroxide (H_2O_2), and reduced glutathione (GSH) as stress biomarkers.

2. MATERIAL and METHODS

2.1. Lichen Material

The *X. parietina* lichen thalli were collected in a rural area far from any urban or industrial area south of Jijel (Algeria) during the spring season 2017. Samples were transported to the laboratory in clean closed boxes. The thalli were then separated from their supports and kept in the laboratory until their use.

2.2. Fluorine Treatment

The lichen thalli of *X. parietina* were incubated in NaF solutions at 0.5, 1.0, 5.0, and 10.0 mM concentrations at room temperature in comparison with a control test which consists of a treatment in distilled water. These solutions were then kept at room temperature for 0, 24, 48, and 96 h in the dark. After treatment and before each analysis, the samples were washed three times with distilled water to remove excess NaF solutions attached to thalli surfaces.

2.3. Chlorophyll Analysis

Chlorophyll a (C_a), chlorophyll b (C_b), and total chlorophyll (C_{a+b}) contents were assayed according to the method described by Lichtenthaler (1987). The fresh lichen sample was macerated in 80 % acetone, and the maceration extract was then filtered and read at 663 nm and 645 nm using a spectrophotometer. Chlorophyll contents were calculated using the following equation:

$$C_a = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$C_b = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$C_{a+b} = 20.2 \times A_{645} - 8.02 \times A_{663}$$

Where A_{663}, A_{645} absorbance at 663 and 645 nm, respectively. Results were expressed in $\mu g.g^{-1}$. To measure the physiological activity of algal cells, the chlorophyll a/b ($c_{a/b}$) ratio was calculated.

2.4. Proteins Assay

Protein contents were tested using Bradford's method (1976). 100 mg of fresh weight lichens were homogenized in 2 ml of 0.05 M phosphate buffer pH 6.8 and centrifuged for 20 min at 12000 t/min at 4 °C. An amount of 2 ml of Bradford's solution was added to 50 µl of supernatant. After 10 min, a reading of 595 nm was recorded. BSA's equation ($y = 28.9$ x, $R^2 = 0.9911$) was used to quantify protein levels ($mg.g^{-1}$).

2.5. Catalase Activity Assay

Catalase activity was measured using the Chance and Maehly's method (1955). An amount of 50 mg fresh weight of lichens was homogenized in 2 ml of 0.05 M phosphate buffer at pH 7, and then centrifuged at 15000 t / min at 5 °C for 20 min. 50 µl of the supernatant was added to 2.95 ml of 0.015 M H_2O_2 in the phosphate buffer. A first reading was taken at 240 nm right away, and a second was taken 3 min later. The following formula was used to calculate catalase's enzymatic activity:

$$k = 2.303 / T \times \log (A_1/A_2)$$

of which:

K: the reaction rate constant**.**

T: Time interval in min.

A_1: Absorbance at t = 0.

A_2: Absorbance after 3 min.

Results were expressed in IU/g of proteins.

2.6. H_2O_2 Assay

H_2O_2 concentration was assayed according to the method described by Sagisaka (1976). An amount of 2 ml of 5 % trichloroacetic acid (TCA) was used to homogenize about 1g of fresh lichen material. The resultant mixture was centrifuged for 20 min at 0 °C at 14000 g. 1.6 ml of supernatant was mixed with a mixture of 0.4 ml TCA (50 %), 0.4 ml ferrous ammonium sulfate (1 %) and 0.2 ml of thio potassium cyanate (1 %). The amount of H_2O_2 in the supernatant was calculated using the optical density at 480 nm. The concentration of H_2O_2 ($mmol.g^{-1}$) was calculated using an equation based on known H_2O_2 standard concentrations ($y = 0.1864 \times + 0.2281$, $R^2 = 0.09691$).

2.7. GSH Assay

GSH assay was carried out by the colorimetric method described by Ellman (1959). GSH is oxidized by producing thionitro-benzoic acid (TNB), which absorbs at 412 nm, in the presence of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB). Lichen thalli were homogenized in 50 mM phosphate buffer (pH 6.5) and centrifuged at 12000 g for 15 min at 4 °C. The absorbance at 412 nm of a combination containing 100 µl of supernatant and 1200 µl of DTNB solution was measured. The results were represented in $mmol.g^{-1}$ using an equation based on known GSH standard concentrations ($y = 0.2012 \times + 0.3852$, $R^2 = 0.9573$).

2.8. Statistical Analysis

Three repetitions were performed at each concentration, so that we could calculate the standard deviation (SD). The statistical study was performed using the ORIGIN 6.0 system using the test univariate variance (one way ANOVA). For this study, the results were expressed as mean ± SD. The difference was considered to be not significant when $p > 0.05$ (NS), significant when $0.01 < p < 0.05$ (*), very significant when $0.001 < p < 0.01$ (**), and highly significant when $p < 0.001$ (***).

Correlation matrices between NaF and different studied parameters were analyzed by STATISTICA Version 10 software.

3. RESULTS

3.1. Chlorophyll Contents Variations

Variations in C_a, C_b, and C_{a+b} contents in *X. parietina* are shown in Figure 1 (a, b and c, respectively), whereas, $C_{a/b}$ ratio variations are presented in Table 1.

Figure 1. Chlorophyll content variations in *X. parietina* after treatment of thalli by NaF solutions, (a): C_a, (b): C_b, (c): C_{a+b}

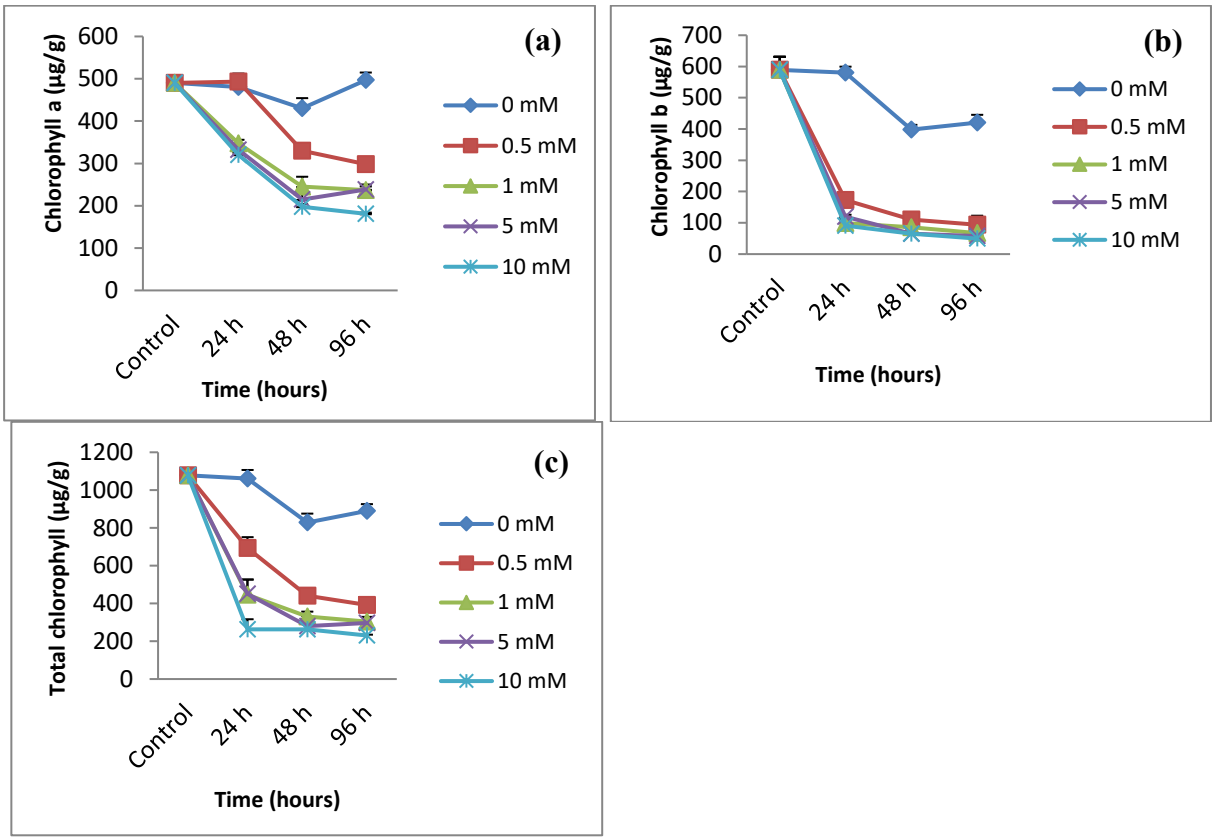


Figure 1 (a) shows a significant decrease in C_a content as a function of different concentrations of NaF ($p = 0.0052^{**}$) and as a function of exposure time ($p = 0.0031^{**}$), variations in C_a levels are not significant between 48 h and 96 h of exposure time ($p > 0.05$).

From the Figure 1 (b), it was noticed that all the concentrations of NaF exhibit the same effect on C_b content, where a very significant decrease was observed ($p = 0.007^{**}$). According to the exposure time, a significant decrease in the content of C_b was also noted in the 24 h following the treatment ($p = 0.0037^{**}$), between 48h and 96 h of treatment, C_b content variations were not significant ($p = 0.755^{NS}$).

Figure 1 (c) shows that the decrease in C_{a+b} content is significant as a function of exposure time ($p = 0.0012^{**}$) as well as a function of NaF concentrations ($p = 0.020^{*}$).

The results presented in Figure 1 allowed us to deduce that the variations in chlorophyll contents (C_a, C_b, and C_{a+b}) in *X. parietina* under NaF stress are significant as a function of exposure time as well as a function of NaF concentrations. From the results presented in Table 1, it was noted a significant increase of $C_{a/b}$ ratio ($p = 0.00572^{**}$). This increase explains well that C_b is the most affected by fluorine compared to C_a.

Table 1. Chlorophyll a/b ratio variations in the lichen *X. parietina* after treatment of thalli by NaF solutions.

	0 mM	0.5 mM	1 mM	5 mM	10 mM
0 h	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02
24 h	0.82 ± 0.02	2.86 ± 0.02	2.5 ± 0.02	2.77 ± 0.1	3.51 ± 0.02
48 h	0.9 ± 0.07	2.98 ± 0.08	2.84 ± 0.04	3.27 ± 0.02	3.03 ± 0.07
96 h	1.18 ± 0.05	3.17 ± 0.02	3.52 ± 0.03	4.13 ± 0.03	3.65 ± 0.05

The data in the table are represented as the mean ± SD.

3.2. Effect of Fluorine on Proteins, Catalase, H_2O_2, and GSH Contents

Under fluorine stress, proteins contents decrease (Figure 2), whereas catalase, H_2O_2 and GSH contents increase (Figures 3, 4, and 5, respectively).

Figure 2. Protein content variations in *X. parietina* after treatment of thalli by NaF solutions.

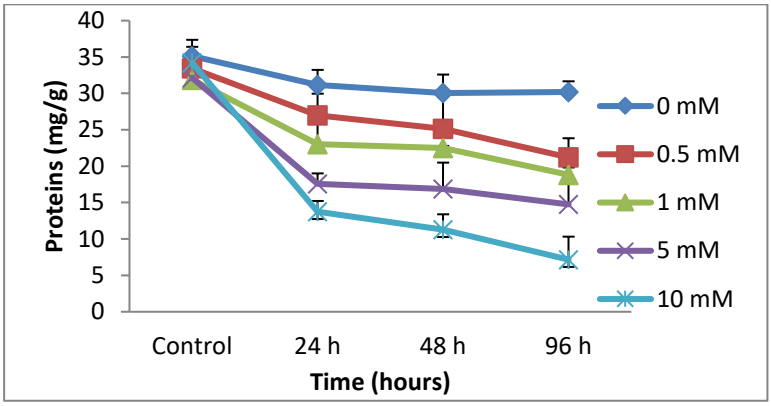
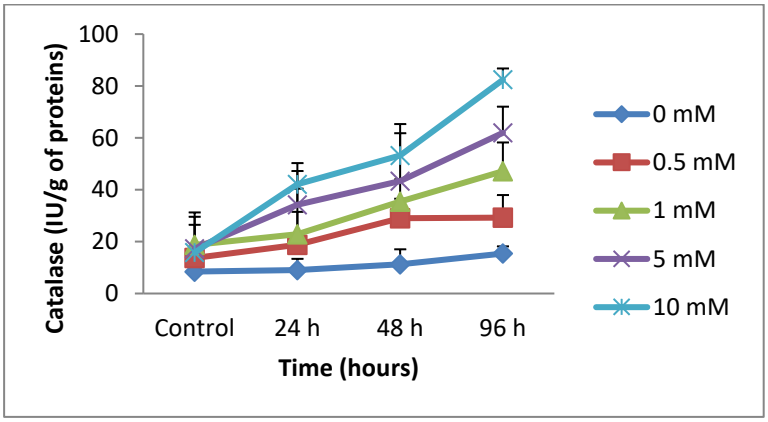


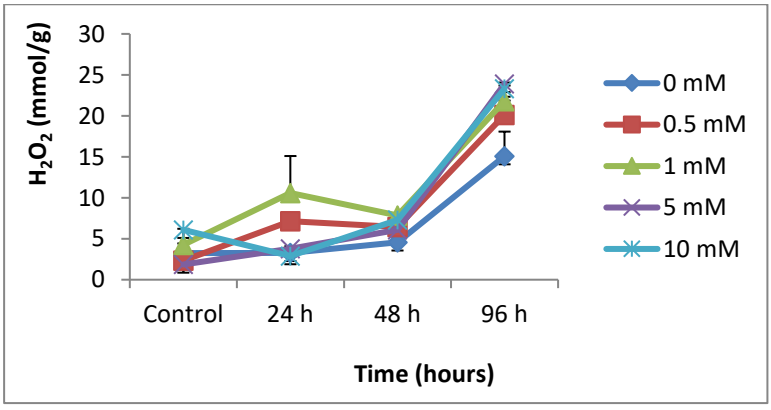
Figure 2 shows that protein levels are affected by NaF, with a significant decrease at all concentrations ($p = 0.0240^{*}$), and a significant decrease was also noted within 24 h of treatment ($p = 0.0062^{**}$); however, between 24 h and 96 of exposure time, the decrease in protein contents was not significant ($p > 0.05^{NS}$).

Figure 3. Catalase activity variations in *X. parietina* after treatment of thalli by NaF solutions.



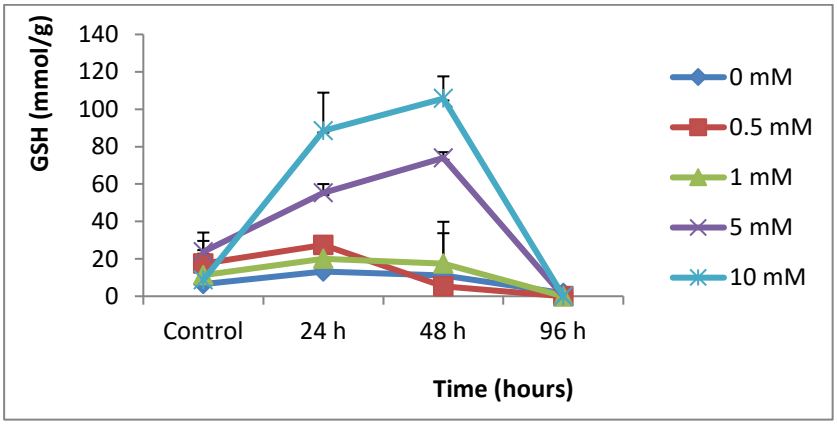
According to Figure 3, it was found that the expression of the catalase increases with increasing concentration ($p = 0.047^{*}$), and increasing exposure time of NaF ($p = 0.045^{*}$).

Figure 4. H_2O_2 content variations in *X. parietina* after treatment of thalli by NaF solutions.



According to the results presented in Figure 4, it was noticed that the variations of H_2O_2 concentrations are not significant either according to the different concentrations of NaF (p = 0.95^{NS}), or after the 48 h which follow the treatment (p = 0.16^{NS}), however, a significant increase was observed after 96 h of treatment (p = 0.017^{*}).

Figure 5. GSH content variations in *X. parietina* after treatment of thalli by NaF solutions.



From the data presented in Figure 5, it was noted that the variations in GSH content in thalli treated with low concentrations of NaF (0.5 mM and 1 mM) are negligible. Whereas, the high concentrations (5 mM and 10 mM) caused a significant increase after 24 h and a non-significant increase after 48 h of treatment (p = 0.64^{NS}). However, a significant decrease in the GSH content was noted between 48 h and 96 h of exposure time (p = 0.02^{*}) with complete degradation after 96 h of treatment.

3.3. Correlation Analyzes

Correlation matrices between NaF and different studied parameters are presented in Table 2. From the data presented in Table 2, the statistical analysis results show a significant negative correlation between C_a, C_b, C_{a+b} and exposure time of NaF and between proteins and increasing concentrations of NaF. A significant positive correlation was noted between $C_{a/b}$ ratio and exposure time of NaF, catalase and exposure time to increasing concentrations of NaF, H_2O_2 and exposure time of NaF, and between GSH and increasing concentrations of NaF. However, a non-significant negative correlation was noted between GSH and 48 h to 96 h of exposure of NaF.

Table 2. Correlation matrices between NaF and C_a, C_b, C_{a+b}, $C_{a/b}$, proteins, catalase, H_2O_2, and GSH contents in *X. parietina*

Correlation matrices	Correlation dependency	r	p	Significance
NaF / C_a	Time (0 - 48 h)	-0.785	< 0.001	***
NaF / C_b	Time (0 - 24 h)	-0.955	< 0.001	***
NaF / C_{a+b}	Time (0 - 48 h)	-0.899	< 0.001	***
NaF / $C_{a/b}$	Time	0.818	< 0.001	***
NaF / proteins	Concentration	-0.872	< 0.001	***
NaF / catalase	Time and concentration	0.784	< 0.001	***
NaF / H_2O_2	Time (48 -96 h)	0.949	< 0.001	***
NaF / GSH	Concentration	0.969	< 0.001	***
	Time (48 - 96 h)	-0.6	0.06	NS

4. DISCUSSION

Compared with the control test, and depending on the increase of concentration and exposure time to NaF, our results show a significant decrease in C_a, C_b, and C_{a+b} contents in *X. parietina*. Zhao *et al*. (2021) found the same thing, indicating that high cadmium concentrations affect photosynthesis in *Sassafras* seedlings. Wang *et al.* (2021) also found that the contents of C_a, C_b, and C_{a+b}, decrease in tall fescue under lead stress. Significant decrease of total chorophyll content was also observed in *Lonicera japonica* Thunb. in response to 150 mg kg^{-1} or 200 mg kg^{-1} of cadmium (Li *et al*., 2022).

Photosynthesis and respiration are the processes most affected by fluoride (Sharma & Kaur, 2018). According to the results of statistical analysis presented in Table 2, it was noted a significant decrease of C_a, C_b, and C_{a+b} contents correlating with exposure time to NaF (r = -0.785, $p < 0.001$; r = -0.955, $p < 0.001$ and r = -0.899, $p < 0.001$, respectively). Our results are in the same line with those obtained by Chakrabarti *et al.* (2014) who reported that chlorophyll decreased in paddy (*Oryza sativa* L.) with increasing fluoride treatment. Mondal (2017) also found pigment degradation in four widely cultivated rice (*O. sativa*) varieties treated to 5, 10, and 20 mg dm^{-3} NaF. An other study carried out by Iram and Khan (2016) showed that C_a, C_b, and C_{a+b} decreased in *Abelmoschus esculentus* (L.) Moench under NaF stress. Fan *et al.* (2022) also found a significant decrease in chlorophyll content after high concentration of NaF treatment in tall fescue (*Festuca arundinacea* Schreb). Our results show a significant increase of $C_{a/b}$ ratio correlating with exposure time to NaF (r = 0.818, $p < 0.001$). These results allowed us to conclude that C_b is more affected than C_a in thalli treated by NaF and the same results were obtained by Purnama *et al*. (2015), who showed a significant decrease in C_b content in Seagrass under lead stress, even though they also found that C_b was more affected than C_a as a result of lead effect.

Plants vary their protein composition for rebuilding, tolerance, resistance, and responsiveness to stressful situations (Amnan *et al.*, 2022). Our results show a significant decrease in protein contents in *X. parietina* correlating with increasing concentrations of NaF (r = -0.872, $p < 0.001$). The same result was obtained by Chetia *et al*. (2021), who found a decrease in total protein contents to correlate with Pb, Cd, Zn, Cu, Co, Ni, and Cr in lichens growing in differently polluted areas. Khan *et al.* (2021) found a drop in total protein contents in cultivated rice grown in lead-contaminated soil. Sharma *et al.* (2019) also showed significant reduction ($p \leq 0.05$) in protein content in *Spirodela polyrhiza* (L.) Schleiden under treatment with fluoride at all the exposure periods (24, 72, 120 and 168 h). According to Szostek and Ciecko (2017), the decrease in total protein content caused by fluoride can be explained by channeling degraded products towards metabolic pathways for energy and stress management. On the other hand, Souahi *et al.* (2021) found an increase in total protein contents in *Triticum durum* Desf. leaves and roots after treatment with 0.3 and 0.6 g/l lead acetate.

Plants increase the activities of antioxidant enzymes like catalase to trap ROS and detoxify their effects (Lei *et al.*, 2022). Ours results show that the expression of catalase in *X. parietina* increases correlating with increasing concentrations of NaF (r = 0.784, $p < 0.001$). Mondal (2017) reported similar results, demonstrating that catalase activity increased with increasing fluorine concentrations in four widely cultivated rice (*O. sativa*). Elloumi *et al*. (2017) also showed that increased catalase activity is one of the indices of oxidative stress induced by fluoride air pollution in *Eriobotrya japonica.* AL-Zurfi *et al.* (2021) found the same results, indicating that *Hydrilla verticillata* responds to cadmium stress by steadily increasing the catalase enzyme concentration. Our results are likewise consistent with those of Abu-Muriefah (2015) and Khan *et al*. (2021), who found that catalase levels increase significantly in plants exposed to lead. Sharma and Kaur (2019) also found a significant increase of catalase activity in *Spirodela polyrhiza* under fluoride stress at high concentration compared to control at a very

first exposure period of 24 h. In addition, Sharma *et al*. (2019) showed that fluoride treatment significantly increased catalase activity in exposed *S. polyrhiza* fronds when compared to control during all exposure periods (24, 72, 120 and 168 h). Ghosh *et al.* (2021) also found that during the ripening of chili fruits, treatment with chitosan and putrescine modulates reactive oxygen species metabolism, and causes an increase in catalase activity. However Chakrabarti *et al.* (2014) found that catalase activity decreased with increasing fluoride treatment. Furthermore, Orabi *et al*. (2015) indicate that the excess of H_2O_2 caused a decrease in the activity of catalase.

Plants create H_2O_2 as one of the ROS in response to abiotic stress (Zhang, 2022). According to Sofo *et al.* (2015), H_2O_2 generation is regarded as a stress marker. H_2O_2 is also necessary for plants to tolerate harsh situations (Černý *et al*., 2018). According to Hung *et al*. (2005), plants have developed complex regulatory mechanisms to adapt to various environmental stresses, the most important of which is to convert the ROS formed into hydrogen peroxide (H_2O_2). The statistical analysis results presented in Table 2 show that H_2O_2 increases correlating with increasing exposure time to NaF ($r = 0.949$, $p < 0.001$). These results are comparable with those of Panda (2007), who investigated the effect of chromium on rice and discovered that this pollutant produces H_2O_2, which is proportional to exposure time and pollutant concentration. Furthermore, those obtained by Liu *et al*. (2010), who found that contents of H_2O_2 decrease in tomato seedlings when the concentration of Mn^{2+} reached 400-600 $\mu molL^{-1}$ under hypoxia stress. Our results are also similarly consistent with those of Liu *et al.* (2021), who found that lead induces the increase of H_2O_2 in edible amaranth under simultaneous stresses of lead from soils and atmosphere, and with those of Li *et al.* (2022) who found higher levels of H_2O_2 in response to high concentration of cadmium (150 mg kg^{-1} or 200 mg kg^{-1} Cd). According to Liu *et al.* (2020), trealose treatment of tomato under cold stress causes elevated H_2O_2 levels as a way of tolerance. Our results show that the accumulation of H_2O_2 is accompanied with the decrease in protein content, the same results were obtained by James *et al.* (2022) who showed a negative correlation between H_2O_2 and proteins in bleuet Northland under hypobaric storage.

GSH increases in plants under stressful conditions (Nahar *et al*., 2017) and it is part of the adaptation strategies used by plants to combat and tolerate stressful conditions (Gong *et al*., 2018). The results obtained show that the GSH content increases correlating with increasing concentrations of NaF ($r = 0.969$, $p < 0.001$) and the same result was obtained by Li *et al*. (2022) who showed that in response to 150 mg kg^{-1} or 200 mg kg^{-1} of Cd, the antioxidants GSH increased in *Lonicera japonica* with increasing concentration of Cd, and by Pristupa *et al*. (2021) who found a decrease in GSH content in transgenic plants *Nicotiana tabacum* L. under abiotic stress conditions.

Correlating with exposure time to NaF, our results show a decrease in GSH content between 48 h and 96 h of treatment ($r = -0.6$, $p = 0.06$). According to Cempírková and Večeřová (2018), the long-term stress exposure of the individual species of green algal and cyanobacterial lichen had a significant impact on the antioxidant content resulting from high light stress. Our results are similar with those of Balarinová *et al*. (2014), who discovered that during the first 30-40 min of high light treatment, total GSH increased in two Antarctic lichens (*Usnea antarctica* and *Usnea aurantiaco-atra*), followed by a reduction at 60 min of treatment, and with those of Li *et al*. (2015) who found that increased heavy metal concentrations resulted in a considerable reduction in GSH content in both safflower roots and leaves.

According to our results, the buildup of H_2O_2 is associated with a decrease in GSH content; these results are in the same line with those of James *et al*. (2022), who found that hydrogen peroxide was negatively correlated with GSH in bleuet Northland under hypobaric storage. Arianmehr *et al.* (2022) also investigated the role of GSH in reducing arsenic (As) toxicity in *Isatis cappadocica* DESV. and *Erysimum allionii* exposed to different concentrations (0, 400,

and 800 M) of arsenic for 3 weeks, and discovered that application of GSH increased fresh weight and total chlorophyll while inhibiting H_2O_2 accumulation.

5. CONCLUSION

The results of the present study revealed that NaF stress caused a decrease in chlorophyll and protein contents, and an increase of H_2O_2, catalase, and GSH levels in *X. parietina* correlating with increasing exposure time and/or increasing concentrations of NaF. Furthermore, the obtained results show that C_b is more affected than C_a, and that high concentration of fluorine disturbed the detoxification system, resulting in total glutathione decomposition.

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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

Ouahiba Benhamada: Investigation, Resources, Visualization, Software, Formal Analysis, Methodology and writing original draft. **Nabila Benhamada:** Analysis, Interpretation and Language revision. **Essaid Leghouchi:** Supervision and Validation.

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Review Article

A systematic review on *Hammada scoparia* medicinal plant: Phytochemicals, traditional uses and biological activities

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Abstract: Medicinal plants have played an important influence in the development of human society; they were always at the forefront of all cultures and civilizations as a source of medicine. Medicinal plants are considered rich sources of secondary compounds, and many modern medications are derived from them. *Hammada scoparia* is a medicinal plant that belongs to the Chenopodiaceae family. It is currently found in North Africa (Morocco, Algeria, Libya, and Egypt) and has many medicinal properties used as folk medicine. The present research discusses the phytochemical components and biological activities of *Hammada scoparia*. The corresponding data were collected from various online databases, new research, and published resources. Different secondary compounds from the species have been documented, including flavonoids, tannins, alkaloids, terpenoids, and saponins. Pharmacological research has demonstrated that the major bioactives have antimicrobial, antioxidant, cytotoxic, and antimalarial activities and anticancer, reno-protective, and hepatoprotective effects. In conclusion, this study provides much information about the interests of vegetal species: *Hammada scoparia*.

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1. INTRODUCTION

Herbal medicine is among the most important branches of ethnomedicine everywhere in the world; it is mainly composed of phytochemicals that assist individuals in recovering and enhancing physiological equilibrium (Laib & Djahra, 2022). Plants are generally used to isolate several medications and formulations to treat various diseases. Screening medicinal plants for bioactive components is required as a base for advanced pharmacological research (Balasundari & Boominathan, 2018). The biological activities of medicinal plants are due to natural products and many kinds of secondary metabolites such as flavonoids, tannins, phenols...etc. *(Djahra et al.*, 2018).

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The Chenopodiaceae family is frequently used in traditional medicine and is involved in people's everyday lives (Gelin *et al.*, 2003). It has 98 genera and about 1400 species. They are frequently found in desert areas, semi-deserts, salt marshes, coastal or inland saline, and ruderal environments. Many Chenopodiaceae species are important components of dry or ruderal habitats (Heklau *et al.*, 2012). *Hammada scoparia* is a Chenopodiaceae family species ;it is well-known for its therapeutic properties in traditional North African medicine, such as cancer, hepatitis, inflammation, and obesity prevention (Ezzeddine *et al*., 2016). Considering the value of natural substances in reducing the severity of diseases, the present study focuses on the traditional applications of *Hammada scoparia* and advances in botany, phytochemistry, and pharmacology of this plant.

2. METHODS

To provide a comprehensive phytochemical and ethnopharmacology review on *Hammada scoparia* species, the corresponding data were collected from various online databases: Pubmed (https://pubmed.ncbi.nlm.nih.gov/), Google Scholar (https://scholar.google.com), Scopus (https://www.scopus.com/), Science Direct (http://www.sciencedirect.com/) Elsevier (https://www.elsevier.com/en-xm), Springer (https://www.springer.com/gp) (date of access: 15 September 2021 and revisited on 21 May 2022), and those published resources (Thesis and books) have been systematically reviewed.

The research strategy used in this paper consisted of combining the following keywords: biological activities, antifungal, antimicrobial, antioxidant, wound healing, and mainly, the scientific name "*Hammada scoparia / Haloxylon scoparium*" or the common name "Remth" followed by the desired domain of research (such as Pharmacology or Phytochemistry, etc.), (*Hammada scoparia* and phytochemistry, e.g.).

3. RESULTS and FINDINGS

3.1. Synonyms and Nomenclatures

Hammada scoparia (Pomel) Iljin = (*Haloxylon scoparium* (Pomel) Bge. = *Haloxylon articulatum ssp. scoparium* (Pomel) Batt. = *Arthrophytum scoparium* (Pomel) Iljin) (Bouaziz *et al.*, 2016; Édouard *et al.*, 2010), It is known as "rimth" in Algeria (Taïr *et al*., 2016).

3.2. Taxonomic Classification

Kingdom: Plantae-plants, Planta, Vegetal, plants; Subkingdom: Tracheobionta; Phylum: Spermatophytes; Superdivision: Angiosperms; Division: Magnoliophyta; Class: Magnoliopsida; Subclass: Caryophyllidae; Order: Caryophyllales; Family: Chenopodiaceae; Genus: *Hammada*; Species: *Hammada scoparia* (Pomel) Iljin (Boucherit *et al*., 2018)

3.3. Botanical Discerption

Hammada scoparia (Figure 1) is a medicinal plant that belongs to the Chenopodiaceae family. It is a grey-brown, woody, small shrub that dries to a dark brown or blackish color (El-Shazly & Wink, 2003), varies in form, having a maximum height of 1 m (Hafidha *et al*., 2017). This plant has articulated leaves and solitary and clustered flowers at the tops of the branches, with a vertical and lateral root structure that helps to preserve and protect the soil from erosion (Boucherit *et al*., 2018).

Figure 1. *Hammada scoparia* species (Benkherara *et al*., 2021 ; Lachkar *et al*., 2021; Ozenda, 2004), respectively.

3.4. Ecology and Distribution

This plant grows in salty soils and prefers an arid or semi-arid environment. It is currently found in North Africa (Morocco, Algeria, Libya, Egypt), Lebanon, Syria, Palestine, Western Sahara, southern Spain, and portions of Iran and Türkiye (Karous *et al*., 2020; Nounah *et al*., 2019).

3.5. Mineral Content

The aerial part of *Hammada scoparia* contains significant quantities of Iron, Potassium, Magnesium, Phosphorus, and Sodium, according to mineral content analyses (Table 1). Copper, Calcium, and Strontium are all found in reasonable quantities. Selenium and Zinc are found in trace amounts (Lachkar *et al.*, 2021).

Table 1. The mineral content of *Hammada scoparia***.**

Mineral elements	*Content in mg/kg of dry matter*
Iron	60909.00
Potassium	27452.10
Magnesium	10059.90
Phosphorus	1125.39
Sodium	1054.65
Copper	438.93
Calcium	313.29
Strontium	280.23
Selenium	3.00
Zinc	3.00

3.6. Ethnomedicinal Uses

Hammada scoparia is widely used in traditional medicine in North Africa (Table 2) to prevent many diseases, such as hepatitis, inflammation, and obesity (Ezzeddine *et al*., 2016). Several traditional applications have revealed that extracts of *Hammada scoparia* have anti-cancer and anti-diabetic properties (Bouaziz *et al*., 2016; Boulanouar *et al*., 2013; Hamza *et al*., 2019; Saidi *et al*., 2015; Taïbi *et al.*, 2020). This species commonly treats diseases, such as decoction, infusion, or cataplasm. It treats hypertension, dermatitis, food poisoning, scabies, injury healing, and gastroenteritis (Eddouks *et al*., 2002; El-Hadri, 2019; Karous *et al*., 2020).

Table 2. Ethnomedicinal uses of *Hammada scoparia*

Disease treated	*Plant part*	*Mode of preparation & administration*	*Country practiced*	*Reference*
Diabetes	Aerial parts	Infusion	Morocco	(Lamchouri *et al*., 2012)
	Flowers, leaves	Decoction	_	(Sabrina *et al*., 2014)
Inflammations & wounds	Aerial parts	Powder mixed with oil of olive by external use	Morocco	(Eddouks *et al*., 2002)
Gastric problems	Aerial parts	_	Algeria	(El-Hadri, 2019)
Scorpion stings and snakebites	Aerial parts	_	Algeria	(Boucherit *et al*., 2018 ; Kharchoufa *et al*., 2020)
Mouth diseases & toothache	Leaves	Infusion/ Decoction	_	(Kharchoufa *et al*., 2020)
Scars	Bark	Powder	_	(Bouaziz *et al*., 2016)
Scabies	Aerial parts	Infusion applied locally	Tunisia	(Karous *et al*., 2020)
Appetizer	Aerial parts	Infusion took orally	Tunisia	
Liver cancer	Aerial parts	Decoction	_	(Taïbi *et al*., 2020)
Thyroid disorders	Aerial parts	Powder mixed with honey	Algeria	(Taïbi *et al*., 2021)
Mould	Fruits, branches	Powder mixed with grease	Algeria	(Boulanouar *et al*., 2013)

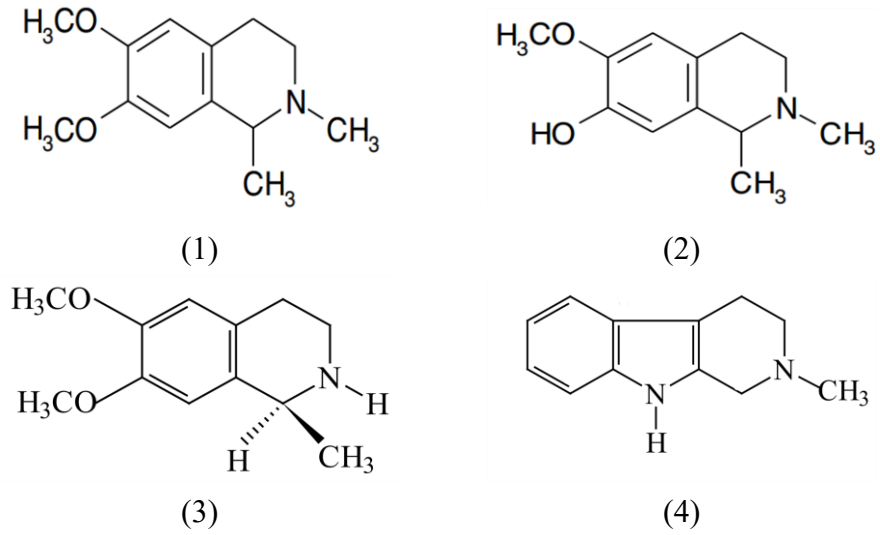
3.7. Phytochemical Constituents

The phytochemical constituents of *Hammada scoparia* have been extensively researched, and the structures of major bioactive molecules have been determined. It is a plant that is especially high in alkaloids and flavonoids. In Figure 2, the structure of the main *Hammada scoparia* alkaloids is presented. Table 3 shows the main compounds that were extracted and identified.

Table 3. Phytochemical compounds identified from *Hammada scoparia.*

Class	*Compound*	*Plant Part*	*Reference*
Alkaloids	Carnegine (1)	Leaves	(Bouaziz *et al*., 2016)
	N-methylisosalsoline (2)		
	Salsolidine (3)	Aerial parts	(El-Shazly & Wink, 2003)
	β-carboline (4)		
Flavanols triglycerides	Isorhamnetin 3-O-β-D xylopyranosyl-(1""3")-α L-rhamnopyranosyl-(1"' 6")-β-D galactopyranoside.	Leaves	(Salah *et al.*, 2002)
	Isorhamnetin 3-O-β-D apiofuranosyl-(1"'2") [α L-rhamnopyranosyl-(1""6")] -β-D galactopyranoside		
	Isorhamnetin3-O-α-L rhamnopyranosyl-(1"'2") [α-L rhamnopyranosyl-(1""6")] -β-D Galactopyranoside		
Flavone	Chrysoeriol	Stems	(Chao *et al*., 2013)
Phenol	Catechol		
Phenolic Acids	Coumaric Acid		
	Cinnamic Acid		
	Caffeoylquinic Acid		
	Catechic acid	Aerial parts	(Benkherara *et al*., 2021)
	Syringic acid		
	Vanillic acid		
	Benzoïc acid		

Figure 2. Structure of major *Hammada scoparia* Alkaloids.



3.8. Biological Activity

The biological activity of the species investigated is shown in Table 4. This section collects published in vitro experimental findings for *Hammada scoparia* species to explain traditional usage and promote future studies on species demonstrating potential in vitro activity.

Table 4. Biological activities of *Hammada scoparia*.

Activity	*Plant part*	*Reference*
Antioxidant	Aerial parts	(Boulanouar *et al*., 2013)
	Leaves & stems	(Bouaziz *et al*., 2016 ; Nounah *et al*., 2019)
Antidiabetic	Aerial parts	(Benkherara *et al*., 2021; Zerriouh, 2014)
Antimicrobial	Leaves & stems	(Bouaziz *et al*., 2016 ; Nounah *et al*., 2019)
	Aerial parts	(Fatehi *et al*., 2018 ; Lamchouri *et al*., 2012)
Antileukemic	Leaves	(Bourogaa *et al*., 2011)

3.8.1. Antioxidant activity

According to research, phenolics are potent scavengers of free radicals and can effectively prevent various diseases (Kamatou *et al*., 2008). Studies demonstrate that areal parts of the plant are an excellent source of polyphenols. The hydroalcoholic extract of *Hammada Scoparia* leaves had excellent antiradical activity (Nounah *et al*., 2019). There was a linear relationship between total phenol content and antioxidant capacity in the evaluated extracts (Bouaziz *et al*., 2016). The extract of *Hammada scoparia* shows antioxidant activity mainly due to reduction and chelation capabilities and sweeping peroxyl radicals (Boulanouar *et al*., 2013).

3.8.2. Antibacterial activity

The use of natural compounds to control infections has been practiced for centuries. Plant exudates and their constituents are one source of natural substances exhibiting antibacterial action (Licá *et al*., 2018). *Hammada scoparia* (leaves and stems) extracts inhibited Gram-positive and Gram-negative bacteria, with average inhibition zones ranging from 8 to 30 mm.

The hydroethanolic extract had the highest antibacterial activity, followed by the methanolic and dichloromethane extracts. *Hammada scoparia* alkaloid extract was effective, with MIC and MBC values ranging from 0.125 to 0.5 mg/ml and 0.25–2 mg/ml, respectively (Bouaziz *et al*.,

2016). The extracts had high anti-bacterial activity against *Citrobacter freundii* and *Acinetobacter baumanii*. The stem extracts were more active against bacteria strains than the leaf extracts, with inhibition zone diameters ranging from 7 to 20 mm for the stem extracts and 7 to 13 mm for the leaf extracts (Nounah *et al*., 2019).

3.8.3. Antidiabetic activity

Hammada scoparia aerial part extract has potential antidiabetic activity. The probable top three extracts demonstrating efficient α-amylase inhibition are Methanolic crude extract, total Flavonoids, and total Alkaloids. *Hammada scoparia* components might be a valuable source of new diabetes drugs used to treat postprandial hyperglycemia with minimal side effects (Benkherara *et al*., 2021; Zerriouh, 2014).

3.8.4. Antileukemic activity

In the search for substances that might reduce cell adhesion-mediated drug resistance (CAM-DR), researchers investigated the effect of *Hammada scoparia* extracts on adherent or suspended leukemic cells. Results demonstrate that *Hammada scoparia* flavonoid fraction and its constituent rutin stimulate apoptosis, particularly in adherent leukemic cells, and reduce CAM-DR (Ezzeddine *et al*., 2016).

4. DISCUSSION

The present research discussed the phytochemical components and biological activities of *Hammada scoparia*. This medicinal species is rich in different secondary compounds, which have been documented, including flavonoids, tannins, alkaloids, terpenoids, and saponins. These compounds are given the plant's broad range of biological activities. Pharmacological research has demonstrated that the major bio-actives have antimicrobial, antioxidant, cytotoxic, and antimalarial activities and anticancer, reno-protective, and hepatoprotective effects. It is thought that the plant is an excellent natural source of secondary metabolites and might be employed in several fields: cosmetics, technology and nanotechnology, nutrition, and alternative medication in the future.

5. CONCLUSION

The results of the present study revealed that NaF stress caused a decrease in chlorophyll and protein contents, and an increase of H_2O_2, catalase, and GSH levels in *X. parietina* correlating with increasing exposure time and/or increasing concentrations of NaF. Furthermore, the obtained results show that C_b is more affected than C_a, and that high concentration of fluorine disturbed the detoxification system, resulting in total glutathione decomposition.

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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

Chaima Benine: Investigation, Data collection, Software, Formal Analysis, and Writing - original draft. **Ali Boutlelis Djahra:** Visualization, Methodology and Supervision. **Laiche Ammar Touhami**: Supervision and Validation. **Abdelkrim Rebiai**: Methodology.

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Research Article

Antioxidant and antimicrobial activities of methanol extracts from *Adonis paryadrica* (Ranunculaceae) – a critically endangered endemic species growing in the Turkish flora

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Abstract: This study was conducted to determine the antioxidant and antimicrobial activities of methanol extract obtained from flower, leaf and root sections of endemic *Adonis paryadrica* (Boiss.) Kandemir & Aytaç stat. nova. naturally growing in the Turkish flora. The most efficient total phenolic compounds and flavonoid contents were obtained from leaf extract at 21.24 mg GAEs (gallic acid equivalent)/g dw and 54.97 mg REs (rutin equivalent)/g dw, respectively. Among the three different sections of this plant, leaf extracts showed the highest Cupric Reducing Antioxidant Power (CUPRAC) effect with 80.28 µmol TEs (trolox equivalent)/g dw. From the three different sections, the methanol extract of the leaf parts demonstrated strong antibacterial activity against *Bacillus subtilis* with a 16.1 mm zone diameter. These valuable and current findings from these precious plants, which constitute natural resources in terms of biodiversity, contribute innovative information to the literature on endemic plant species.

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1. INTRODUCTION

Plants have been preferred as one of the main sources of natural remedies for centuries, and still today, the new compounds of these valuable organisms continue to be documented as natural sources (Egamberdieva and Tiezzi, 2019). Secondary metabolites are the basis of these natural resources. Ranunculaceae family with a wide distribution in the world contributes to these natural resources pool with its secondary compounds such as alkaloids, glycosides derivatives, saponins, and steroids, including bufadienolides and cardenolides (Hao *et al*., 2017; Kuroda *et al*., 2018). The genus of Adonis L., belonging to the Ranunculaceae family, is represented by approximately 40 species in the world. This genus has 9 species and one subspecies in Turkey. Among these 10 taxa, only *Adonis paryadrica* (Boiss.) Kandemir & Aytaç stat. nova. is a rare endemic species for Turkish flora. This species has been collected in 1858 by Tchihatcheff Pierre from Giresun, Turkey for the first time. After a long break, this species was investigated within the scope of biodiversity study by the Ministry of Agriculture and Forestry of the Republic of Turkey in 2018. Within the scope of the project, the presence

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of the species was confirmed in six locations of Şebinkarahisar-Alucra districts in Giresun and one location in Erzincan Munzur Mountains. The distance between locations with semi-acidic soil characteristics is approximately 20 km from the bird's eye view. The taxon is very insufficient in terms of the number of individuals in all of the determined locations and is stuck in a narrow area. Many limiting factors such as grazing pressure, erosion, road construction activities and insect attacks have encountered in some areas where the species lives (Ministry of Agriculture and Forestry, 2018). For all these reasons, it has been reported that the species is endangered and should be evaluated in the Critically Endangered (CR)" (criteria B2 a b (i, iii) of IUCN 2010) category (IUCN, 2010; Kandemir *et al*., 2019).

There are a limited number of studies on phytochemical components of this genus in the literature (Mohadjerani *et al*., 2014; Kuroda *et al*., 2018; Ucuncu *et al*., 2020). Although a study has been reported on the phytochemical and biological activity of ethanol extracts of *A. paryadrica*, however, there is no comprehensive study in the literature on the biological activities of extracts obtained with other solvents up to the best of our knowledge. The aim of this study was to determine the (i) antioxidant activity, (ii) total phenolic compounds, (iii) total flavonoid contents and (v) antimicrobial activity of the methanol extracts of this valuable rare natural resource as well as the ongoing in situ and ex situ conservation studies. All of these obtained results will shed light on the evaluation of bioactive phytochemical constituents of *A. paryadrica* as medically.

2. MATERIAL and METHODS

2.1. Electrochemical Method

All electrochemical measurements were obtained using an electrochemical analyzer i.e. Vertex®One (Ivium) device which includes electrode cell stand. This electrode cell stand consists of a reference (Ag/AgCl; BASi, MF-2052), a counter (platinum wire; BASi, MW-1032) and a working electrodes (glassy carbon electrode (GCE); BASi MF-2012). In order to make the indicator electrode clean, smooth, polishing the surface of GCE with aluminum silica was applied before each measurement. The electrochemical method as square wave stripping voltammetry (SWSV) was used to determine the amount of antioxidants found in the root, stem and leaves of the *A. paryadrica* on GCE. The operating conditions of SWSV were selected as pulse amplitude of 60 mV, frequency of 100 Hz, step potential of 5 mV, accumulation time of 30 s and accumulation potential of 0 mV. For the supporting electrolyte solution, the Britton-Robinson buffer solution at pH 6.0 was used to collect all SWSV data. pH of solutions was adjusted with a Mettler Toledo brand pH meter with an accuracy of ± 0.05. No pre-purification was applied to the samples. The samples of *A. paryadrica* were prepared for spectrophotometric analysis and used directly.

2.2. Reagents

The analytical standard of rutin was purchased from Aldrich-Sigma. Stock solution for rutin was prepared daily at a concentration of 500 mg/L. Britton Robinson (BR) buffer solution preferred as support electrolyte was prepared with 0.4 M of acetic acid, ortho-phosphoric and boric acid. To adjust the BR buffer solution to pH 6.0, 2.0 M NaOH or 2.0 M HCl solutions were used. Distilled water was used in the whole experimental process.

2.3. Spectrophotometric Methods

The antioxidant activities of the methanolic extracts from plants were expressed as mg trolox equivalent (TEs)/g extract. Details of the spectrophotometric methods can be found in supplementary file (Zengin *et al*., 2015a; Zengin *et al*., 2015b; Apak *et al*., 2006; Kocak *et al*., 2010).

2.4. Preparation of the extracts of *Adonis paryadrica*

Air-dried samples of the aerial parts (2 g) of plants were extracted with 50 ml of methanol for 30 min in a sonication bath at 30 °C. The extracts were filtered and then concentrated under reduced pressure. All samples were stored at –20 °C before using for experiments.

2.5. Activity Test

2.5.1. *Microbial strains*

MeOH extracts of flower, leaf and root parts of the *A. paryadrica* were individually tested against six Gram-positive, seven Gram-negative bacteria and a fungus. Detailed information about the strains of bacteria and fungus were given in the supplementary file.

2.5.2. *Disc diffusion assay*

The assay was performed by following the protocols of the Clinical and Laboratory Standards Institute (2014) and The European Committee on Antimicrobial Susceptibility Testing (2013). Details of the disc diffusion analyses were also specified in the supplementary file.

2.5.3. *Microdilution assay*

Minimum Inhibitory Concentration (MIC) was tested for four Gram-positive and two Gram-negative bacteria. MeOH extracts of flower part of the *A. paryadrica* were individually tested against only three Gram-positive bacteria. For this purpose, the flower part of this species was individually tested against four Gram-positive bacteria and two Gram-negative bacteria. Detailed information about the MIC values were given in the supplementary file.

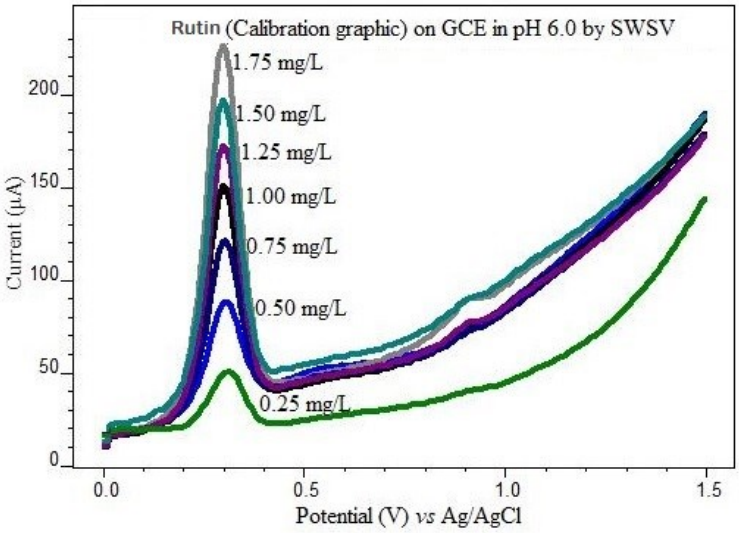
2.6. Statistical Analysis

All spectrophotometric and electrochemical tests were performed in triplicate and the results were expressed as mean with standard deviation (mean ± SD). The electrochemical analyses of antioxidant capacity of *A. paryadrica* samples were carried out by calibration method performed under the optimum condition for analytical standard i.e. rutin. Statistical significance between data was determined by using Tukey's honestly significant difference post hoc test with $\alpha = 0.05$ and ANOVA (one-way analysis of variance) test. Statistical calculations were carried out by using SPSS v. 22.0 software.

3. RESULTS and DISCUSSION

Square wave stripping voltammetry (SWSV) is generally one of the most preferred electrochemical methods for sensitive, selective, cheap and fast analysis of substances that are electroactive types such as phenolic compounds, flavonoids, vitamins, drugs and pesticides (Demir and İnam, 2014; Demir, 2019; Yıldırım *et al*., 2020; Demir and Silah, 2020; Inam *et al*., 2020; Demir *et al*., 2021). Therefore, firstly, SWSV experimental conditions were selected for the rutin, which is one of the standard antioxidants. The operating conditions of SWSV were preferred as pulse amplitude of 60 mV, frequency of 100 Hz, step potential of 5 mV, accumulation time of 30 s, accumulation potential of 0 mV and BR buffer solution at pH 6.0 on GCE. Two oxidation peaks were obtained at 320 mV and 910 mV for rutin by SWSV on GCE in pH 6.0 BR buffer solutions (Figure 1).

Figure 1. Calibration graphic of rutin by SWSV on GCE.

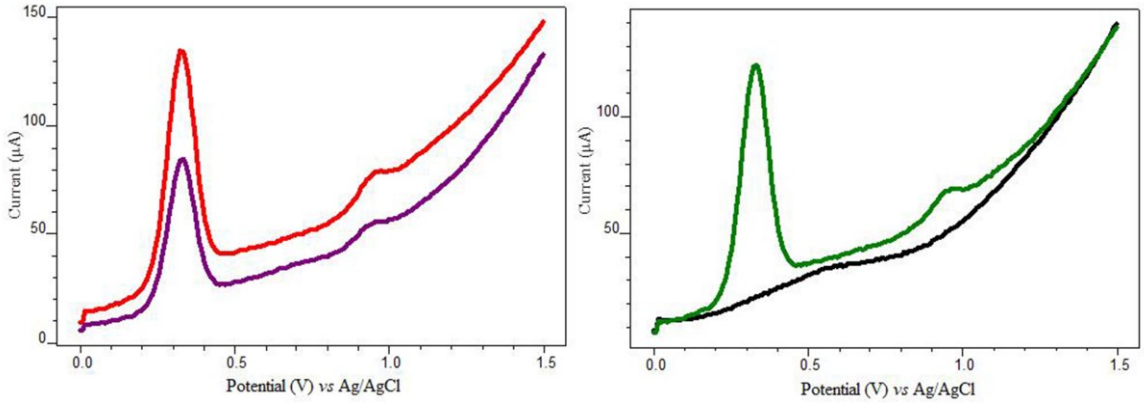


Then, under these conditions, based on the anodic peak at approximately 350 mV, calibration was created with standard addition between 0.25 mg/L and 1.75 mg/L by SWSV method. Linear calibration equation for the rutin agent was obtained by plotting peak signal on SWS voltammograms versus concentrations. The obtained equation on the linear calibration graphics for the rutin is as follows:

$$I_p\ (\mu A) = 105.15\ C\ (mg/L) + 8.\ 247\ R2 = 0.9961 \text{ for the rutin}$$

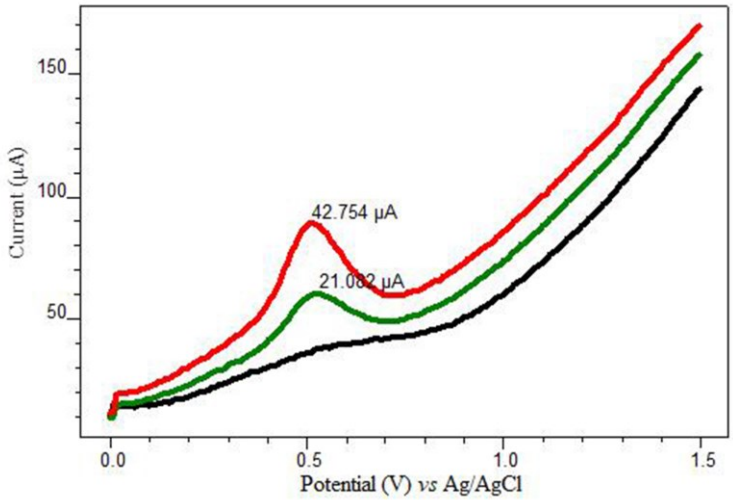
Under the conditions set for standard antioxidant substance, SWS voltammograms of real samples such as for the stem and leaves of the *A. paryadrica* were obtained on GCE in pH 6.0 BR buffer solutions (Figure 2a and 2b).

Figure 2. SWS voltammograms for the a) Flower b) Leaf sample on GCE in pH 6.0 BR buffer solutions.



Two oxidation peaks were obtained at 350 mV and 675 mV of real samples on the glassy carbon electrode (GCE) under SWSV test conditions. The peak potential values obtained for these two samples are almost identical to the peaks exhibited by rutin. Therefore, the amount of antioxidants contained in these two samples can be easily calculated in terms of rutin equivalent. Here, the first anodic peak which is high intensity peak and well-defined at approximately 350 mV was referenced. As a result of three replicate measurements for the 0.05 ml samples, average peak currents for flower samples were found to be 62.29 ± 2.78 µA, while this value was calculated as 95.88 ± 3.56 µA for leaf samples. The total antioxidant capacity (TAC) for the 1 g extract of flower and leaf samples as rutin equivalent were found as 257.0 mg/L and 416.5 mg/L, respectively. The peak obtained for root samples differs from the peak potential values obtained for flower and leaf (Figure 3).

Figure 3. SWS voltammograms for the root sample on GCE in pH 6.0 BR buffer solutions.



The main reason of this difference is dominance of phenolic compound in the root samples that is different from the rutin. However, since the peak potential of the phenolic compounds is between 0.3 V and 0.5 V, it is possible to give the total amount of antioxidants in the root sample as rutin equivalent. According to the results of three replicates for the 0.05 ml extract sample, the peak flow value was found to be 21.425 ± 0.672 µA. The TAC in root sample was calculated as rutin equivalent of 88.29 ± 2.77 mg/L (Table 1).

Table 1. Total antioxidant amounts in equivalent rutin in plant samples by SWSV.

Method	Samples	Total antioxidant capacity Equivalent rutin
SWSV	Flower	257.0 ± 11.45^{b} mg/l
	Root	88.29 ± 2.77^{c} mg/l
	Leaf	416.5 ± 14.67^{a} mg/l

Antioxidant quantification of the endemic plant *Draba cemileae* (Karaer) in leaf, root and stem samples were carried out with the electrochemical method by Cuce *et al*. (2021). The electrochemical measurements are compatible with the data obtained by spectrophotometric-based methods such as traditional Cupric Reducing Antioxidant Power (CUPRAC) reducing, Ferric Reducing Antioxidant Power (FRAP) reducing, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferrous ion chelating (Cuce *et al*., 2021). Moreover, electrochemical methods have been successfully performed to determine the total antioxidants in many products such as fruit juices, coffee samples and many different foodstuffs (Yıldırım *et al*., 2020; Demir *et al*., 2021; Öztürk *et al*., 2021). Therefore, the antioxidant capacities of *A. paryadrica* were investigated in detail by electroanalytical methods, which is a fast, inexpensive, simple, accurate and reliable new antioxidant method.

3.1. Spectrophotometric Methods

3.1.1. *Chemical composition*

The yields of the MeOH extracts obtained from the flower, leaves and roots of *A. paryadrica* were shown in Table 2. The highest yield percentage was obtained from the flower extract with 18.76% followed by the root (16.59%) and leaf (13.80) extracts, respectively.

The spectrophotometric analysis data were also given in Table 2. Leaf parts were found to be the richest in terms of total phenolic substances (21.24 mg GAEs/g) and total flavonoid content (54.97 mg REs/g). The root parts were found poorest in terms of both phenolics (18.59

mg GAEs/g), and flavonoids (0.54 mg REs/g) than other parts. Statistical analyzes showed that the phenolic profiles of flower and root extracts were not significantly different from each other ($p < 0.05$). In addition, when the flavonoid profiles were compared statistically, it was determined that the flower, leaf and root extracts were significantly different from each other ($p < 0.05$).

Table 2. Extraction yield, total phenolic and flavonoid contents of the methanol extracts from *A. paryadrica.*

Samples	Yield (%)	Total phenolics (mg GAEs/g extract)	Total flavonoids (mg REs/g extract)
Flower	18.76	19.17 ± 0.23^{b}	32.42 ± 0.01^{b}
Root	16.59	18.59 ± 0.37^{b}	0.54 ± 0.02^{c}
Leaf	13.80	21.24 ± 0.14^{a}	54.97 ± 0.51^{a}

Within each column, means sharing the different superscripts show comparison between the extracts by Tukey's test at $p < 0.05$. GAEs and REs, gallic acid and rutin equivalents, respectively

Evaluation of the biological activities of valuable natural metabolites of plants is one of the most important research areas today and there are many studies carried out in this field (Cüce *et al*., 2017, 2019; Cüce and Basançelebi, 2021; Sarikurkcu *et al*., 2021). The different techniques and methods used in these studies or the different extraction techniques and solvents for the same species can be shown among the reasons for the results obtained from the studies (Jahanban-Esfahlan *et al*., 2019; Ucuncu *et al*., 2020; Sarikurkcu *et al*., 2020). Due to this situation, the researchers preferred ethanol extracts for *A. paryadrica* in previous studies and reported that they obtained similar results in terms of total phenolic contents with the data in our study (Ucuncu *et al*., 2020). The strength of this study is that the percentage yield and total flavonoid content values were not given in the previous study on *A. paryadrica.*

3.1.2. *Antioxidant activity*

The data regarding the antioxidant activity potentials of the extracts are summarized in Table 3. The extracts differed in terms of radical scavenging capacities. DPPH radical scavenging capacity order of extracts were leaf>flower>root and ABTS radical scavenging capacity order of extracts were root>leaf>flower (Table 3). Based on these data, the highest DPPH radical scavenging effect was found to be 38.26 mg/ml in leaf extracts, while the most effective ABTS radical scavenging effect in root extracts was calculated as 44.78 mg/ml. The DPPH radical scavenging capacity of root extracts (12.58 mg/ml) created a statistically significant negative difference from others. ABTS radical scavenging capacities of the leaf and flower extract were determined to be 39.71 and 33.70 mg/ml, respectively, which is an indicator of the statistical difference between them ($p < 0.05$).

Similar scenarios were experienced in terms of the CUPRAC and FRAP reducing powers of the extracts. While the leaf extract gave the most effective CUPRAC reducing power activity with 80.28 mg/ml, the root extracts had 45.05 mg/ml FRAP reducing power. In terms of CUPRAC reducing power activity, leaf extracts were followed by flower (66.54 mg/ml) and root (49.59 mg/ml) extracts, respectively. On the other hand, in terms of FRAP reducing power activity, root extracts were followed by leaf with 42.11 mg/ml and flower (34.17 mg/ml) extracts, respectively (Table 3).

Table 3. Radical scavenging activities of methanolic extracts from *A. paryadrica*.

Samples	DPPH radical (TEs/g extract)	ABTS cation radical (TEs/g extract)	CUPRAC reducing (TEs/g extract)	FRAP reducing (TEs/g extract)
Flower	30.94 ± 0.11^{b}	33.70 ± 0.20^{c}	66.54 ± 0.25^{b}	34.17 ± 0.53^{c}
Root	12.58 ± 0.19^{c}	44.78 ± 0.01^{a}	49.59 ± 0.41^{c}	45.05 ± 0.01^{a}
Leaf	38.26 ± 0.03^{a}	39.71 ± 0.14^{b}	80.28 ± 0.25^{a}	42.11 ± 0.24^{b}

Within each column, means sharing the different superscripts show comparison between the extracts by Tukey's test at $p < 0.05$. TEs, trolox equivalents

Antioxidant studies of species belonging to the genus Adonis are limited in the literature (Mohadjerani *et al*., 2014; Ucuncu *et al*., 2020; Guo *et al*., 2022). In antioxidant studies of *A. paryadrica* with only ethanol extracts, researchers used different parts of the plant and obtained effective results on the gallic acid equivalent (Ucuncu *et al*., 2020). The data obtained from this study also showed that the difference in the extract had an effect on the antioxidant properties of the Trolox equivalent.

3.2. Antimicrobial Activity

According to the results of the agar disc diffusion test, some bacteria were inhibited by the flower and leaf extracts. The root extract of the plant and negative control (20% DMSO) did not show any antimicrobial activity against bacteria. The inhibition zones against the tested bacteria ranged from 8.21 to 16.1 mm. The highest inhibition zone was obtained from the leaf extract against *B. subtilis* with 16.1 mm (MIC = 62.5 µg/mL). Among the bacteria whose inhibition value was obtained, E. faecalis had the lowest value with a zone diameter of 8.2 mm (MIC = 125 µg/mL). Flower extract was effective as antibacterial against S. aureus and *B. subtilis* and S. faecalis with 9.2 mm, 15.2 mm and 10.2 mm inhibition zone, respectively. MIC values for the mentioned these bacterial species were calculated as 125, 31.25 and 62.5 125 µg/mL, respectively. None of the flower leaf and root extracts showed any inhibition effect on *C. albicans*. Methanolic extracts of *A. paryadrica* demonstrate antimicrobial activity against two Gram-negative and four Gram-positive bacteria according to the disc diffusion assay (Table 4).

Diameter of inhibition zone including disc diameter of 6 mm by the agar dics diffusion method at a concentration of 10 µL of extract/disc. Ofloxacin (10 µg/disc) (OFX), netilmicin (30 µg/disc) (NET30), sulbactam (30 µg) + cefoperazone (75 µg); (105 µg per disc) (SCF) were used as reference antibiotics. Dimethyl sulfoxide (DMSO) (20%) was used as negative control (N.C.). MIC (minimal inhibition concentration) was calculated as µg/ml. The values are the average ± standard deviation of three determinations ($p < 0.05$). - = not detected. Values with different letter(s) in the same line(s) were significantly different ($p < 0.05$)

Due to the fact that microbial contamination is one of the most threatening elements of today, research is focused on this subject. Researchers carry out antimicrobial studies on each pathogenic microorganism against the risks of future epidemics, and herbal phytochemicals come first as a source (Tepe *et al*., 2005; Rios and Recio, 2005; Cüce and Basançelebi, 2021). In the study conducted on the antibacterial effects of ethanol extracts of *A. paryadrica*, the researchers obtained the highest zone diameter of 14 mm from the flower extract on *Yersina pseudotuberculosis* (Ucuncu *et al*., 2020). These researchers generally reported that flower extracts showed more effective antibacterial activity on different bacterial species.

On the other hand, the calculation of the highest inhibition zone on *B. subtilis* with 16.1 mm reveals the difference in the present study. According to these results, the leaf extract of *A. paryadrica* can be said more effective than other parts in terms of the antibacterial activity.

Table 4. Zones of growth inhibition (mm) showing antimicrobial activity of *A. paryadrica*

Bacteria	Disc Diffusion (mm)			MIC (µg/mL)			N.C.	Standard Antibiotic Discs		
	Flower	Leaf	Root	Flower	Leaf	Root		OFX	NET30	SCF30
Gram Negative Bacteria										
K. pneumoniae	-	8.5 ± 0.2^{d}	-	-	125	-	-	16.7 ± 0.6^{c}	18.1 ± 0.7^{b}	21.6 ± 0.8^{a}
E. coli	-	-	-	-	-	-	-	26.6 ± 1.1^{b}	16.6 ± 0.8^{c}	29.0 ± 0.5^{a}
S. marcescens	-	-	-	-	-	-	-	26.6 ± 0.7^{b}	20.0 ± 0.6^{c}	28.4 ± 0.9^{a}
S. typhimurium	-	-	-	-	-	-	-	26.3 ± 0.2^{b}	19.1 ± 1.5^{c}	28.2 ± 0.0^{a}
P. aeruginosa	-	-	-	-	-	-	-	27.8 ± 0.1^{a}	20.6 ± 0.3^{b}	27.4 ± 0.5^{a}
P. vulgaris	-	9.3 ± 0.3^{c}	-	-	31.25	-	-	29.1 ± 0.6^{a}	16.6 ± 0.8^{b}	29.2 ± 0.9^{a}
S. enterica	-	-	-	-	-	-	-	28.7 ± 0.5^{a}	27.4 ± 0.2^{a}	19.5 ± 0.7^{b}
Gram Positive Bacteria										
S. aureus	9.2 ± 0.2^{d}	9.1 ± 0.2^{d}	-	125	125	-	-	29.1 ± 1.0^{a}	20.6 ± 0.5^{c}	25.1 ± 0.6^{b}
B. subtilis	15.2 ± 0.3^{d}	16.11 ± 0.3^{c}	-	31.25	62.5	-	-	30.4 ± 1.4^{a}	29.3 ± 1.5^{a}	28.5 ± 1.5^{b}
S. epidermidis	-	-	-	-	-	-	-	27.5 ± 1.5^{b}	26.2 ± 1.1^{b}	31.7 ± 0.0^{a}
B. thrungiensis	-	-	-	-	-	-	-	25.4 ± 0.5^{b}	23.6 ± 1.0^{c}	28.6 ± 0.8^{a}
E. faecalis	-	8.21 ± 0.1^{d}	-	-	125	-	-	19.3 ± 0.5^{b}	21.2 ± 1.3^{a}	14.3 ± 0.7^{c}
S. faecalis	10.2 ± 0.2^{d}	11.03 ± 0.3^{c}	-	62.5	62.5	-	-	19.9 ± 0.5^{a}	16.6 ± 0.0^{b}	19.9 ± 0.7^{a}
Fungi										
C. albicans	-	-	-	-	-	-	-	20.5 ± 0.5^{b}	19.8 ± 0.6^{c}	18.7 ± 0.0^{d}

4. CONCLUSION

In this study, innovative and traditional antioxidant detection methods were applied to flower, leaf and root methanol extracts of *A. paryadrica*, an important endemic plant, and effective results were obtained. In terms of total antioxidant capacity, electrochemical and spectrophotometric antioxidant analysis methods have generally shown that the leaf parts of this plant are much more effective. Leaf extracts were found to be more effective than the other parts in terms of antimicrobial analyzes on Gram-negative and Gram-positive bacterial species and a fungus. This study is important in terms of revealing the pharmaceutical properties of different extracts obtained from medicinal and aromatic plants grown in our country and have medicinal value. It is envisaged that these studies will constitute a model for similar studies to be carried out in the near future.

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Declaration of Conflicting Interests and Ethics

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

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Publisher's note

Correction to: *International Journal of Secondary Metabolite,* (2022), https://doi.org/10.21448/ijsm.1071234. The family name in the title of the article has been changed to "Ranunculaceae". The "Asteraceae" family name in the title was incorrect. The original article has been corrected.

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