

Antioxidant and antimicrobial activities of *Ballota nigra* L. subsp. *anatolica*

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ARTICLE HISTORY

Received: Dec. 26, 2024

Accepted: Mar. 22, 2025

KEYWORDS

Antimicrobial activity,
Antioxidant activity,
Ballota nigra,
Total phenolic content.

Abstract: Many ancient and contemporary societies have relied heavily on herbal treatments for medical care. Plant's secondary metabolites have extensively different bioactivity properties. Several species in the genus *Ballota* L. have been shown to have medicinal benefits for various illnesses. *B. nigra* L. from both clinical and experimental investigations provides strong proof that this plant can prevent serious illnesses. External usage of *B. nigra* species is used to treat burns and wounds. Certain subspecies of *B. nigra* are utilized for its aerial parts internally, where they are used to reduce cough, alleviate inflammation, and treat gastrointestinal issues. In this study, ethyl acetate, methanol extracts, and infusion samples of the aerial parts of *B. nigra* subsp. *anatolica* were prepared, and their antimicrobial activity by using microbroth dilution method against six pathogens, total phenolic content by the Folin-Ciocalteu method, and antioxidant effects by the DPPH method was investigated. As a finding, the highest concentration of total phenol (66.8 mg GAE/g ext) was found in the methanol extract. The ethyl acetate and infusion extracts had corresponding total phenol concentrations of 43.2 mg and 49.6 mg GAE/g ext. The methanol extract of the plant exhibited higher antioxidant activity than other extracts, with an IC₅₀ value of 0.516 mg/mL. The infusion samples exhibited no antibacterial action, and the methanol and ethyl acetate extracts' MIC values ranging from 97 to 3125 µg/mL. It is possible to conclude that yeast species are more susceptible to plant extracts than bacteria species with low MIC values.

1. INTRODUCTION

Plants have long been considered one of the world's most valuable natural resources. Human illnesses have been treated with natural materials extracted from a variety of sources, including plants. Many natural compounds have biological activity that can be used in drug design and discovery. Fragments formed from natural products and fragment-like natural products will be important in the next generation of drug development since they can be combined with other molecules (Morteza-Semnani & Ghanbarimasir, 2019). The Lamiaceae family includes the genus *Ballota* L., which has roughly ninety species and is found all over the world (Kazemizadeh *et al.*, 2009). Twelve species and eight subspecies of *Ballota* are found in Turkey (Davis, 1982; Güner *et al.*, 2012). *Ballota* species are small shrubs or perennial herbs with

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simple or branching hairs, and toothed, petiolate leaves; the inflorescence, which can be either racemoid or thyrsoid, occasionally contains long, spinose bracteoles (*Acanthorsium* sect.); the calyx is primarily campanulate, ranging in color from purple to white (Rosselli et al., 2019).

B. nigra L. known as "yalancı ısırgan" in Türkiye, is used to cure gut disorders, prevent nausea and vomiting, and exhibit sedative characteristics in situations of anxiety and tension (Demirezer et al., 2019; Duke et al., 2002). Its aerial parts are utilized as infusions, liquid extracts, or tinctures (Duke et al., 2002). Known as "grip otu," *B. nigra* L. subsp. *anatolica* P. H. Davis has been used in traditional medicine for nausea, vomiting, and nervous dyspepsia as well as an antibacterial, anti-inflammatory, antioxidant, and antirheumatic agent (Güner et al., 2012; Demirezer et al., 2019; Tipirdamaz & Guvenc, 2004). It is frequently used for a variety of purposes, most notably as a neuro-sedative treatment. The chemical examination of *B. nigra* revealed the presence of oils, betaines, diterpenes, flavonoids, and phenylpropanoid glycosides. Pharmacological research on *B. nigra* has already revealed the plant's neuro-sedative, depressive, insecticidal, anticholinesterase, and antifeedant properties (Hemanth et al., 2021). By far the most widely used is *B. nigra*, a perennial herb native to central Asia and the Mediterranean region that grows all over Europe. Additionally, New Zealand, Argentina, and the United States have granted it naturalization. *B. nigra* leaves were once employed as a countermeasure against rabid dog attacks. In cases of hysteria and hypochondria, it was employed as a sedative/tranquilizer in the Balkans. Italy also uses it externally for its ability to cure wounds. Internally, it is used for whooping cough, as a sedative, as a spasmolytic for stomach aches and cramps, and to stimulate bile flow in the Balkans. Additionally, it is utilized to alleviate nausea, vomiting, upset stomach, and anxiousness. It is used to treat worm infestation in Moldova by enemas and suppositories. It is used as a pesticide and flea repellent in northern Spain. One of the subspecies of the species, *B. nigra* subsp. *anatolica* is known by many local names in different regions of Türkiye. It has been used as an antiseptic for wounds, burns, and inflammatory skin, as well as a treatment for flatulence, upset stomach, colds, and flu (Pieroni, 2000; Mulas, 2006; Yeşilada et al., 1995).

In this study, ethyl acetate, methanol extracts, and infusion samples of the aerial parts of *B. nigra* subsp. *anatolica* were prepared, and their antimicrobial and antioxidant effects were examined. This study provides a novel contribution to the scientific understanding of *B. nigra* subsp. *anatolica* by offering the first comprehensive evaluation of its antioxidant, phenolic, and antimicrobial properties. The results demonstrate that the methanol extract exhibits remarkable antioxidant activity and phenolic content, surpassing other tested extracts. Furthermore, the findings highlight the greater sensitivity of yeast species compared to bacteria, suggesting the plant's potential for selective antimicrobial applications. This research fills a critical gap in the literature and paves the way for future pharmacological and phytotherapeutic investigations.



Figure 1. Habit of *Ballota nigra* subsp. *anatolica*.

2. MATERIAL and METHODS

2.1. Plant Material

Ballota nigra subsp. *anatolica* was collected from Çanakkale in June 2023. The plant was photographed (Figure 1) in its habitat and described by Dr. Nagehan Saltan using the 'Flora of Turkey and East Aegean Islands' prepared by Davis, 1982. The sample is preserved in the Herbarium at Anadolu University, Eskişehir, Türkiye with Voucher specimen no: ESSE 16310. Extracts were obtained from the aerial parts of the plant using methanol and ethyl acetate. A 5% infusion was prepared from the aerial parts of the plant.

2.2. Antioxidant Activity and Total Phenolic Content

By using the DPPH free radical scavenging effect assay and the technique outlined by Agiel *et al.*, the antioxidant activities of the extracts get from the aerial parts of *B. nigra* subsp. *anatolica* were assessed. The Folin-Ciocalteu reagent (FCR), a technique based on total phenolics, was used to measure the extracts' total phenolic content as milligrams of gallic acid equivalent (Aguel *et al.*, 2024; Singleton *et al.*, 1999).

2.3. Antimicrobial Activity

To monitor antimicrobial activity, the methanol, ethyl acetate extracts, and infusion samples of *B. nigra* subsp. *anatolica* were determined against Gram-positive (*Staphylococcus aureus* ATCC 29213, *Bacillus cereus* NRRL B-3711, *Listeria monocytogenes* ATCC 19111), Gram-negative (*Pseudomonas aeruginosa* ATCC 27853) and *Candida* species (*C. albicans* ATCC 90028 and *C. krusei* ATCC 6258) by the broth microdilution method (CLSI, 2002a; CLSI, 2012). Minimum Inhibitory Concentration (MIC) values were ascertained by the broth microdilution method. The experiments were performed with minor modifications to CLSI standards. Concentrations ranging from 485 to 6250 µg/mL were evaluated using a two-fold serial dilution method. Fresh cultures of microorganisms were made overnight on the proper media (Saboroud Dextrose Broth for yeasts, and Mueller Hinton Broth for bacteria). The McFarland 0.5 standard was followed in the preparation of standard microorganisms, and the number of microorganism colonies per milliliter was adjusted to be 1×10^8 . A 96-well plate was filled with two-fold diluted plant extracts, and 100 µL of standard microorganisms were placed on top of it. For 24 hours, the plates were incubated at 37 °C. The positive control group consisted of the standard antibiotics (Erythromycin and Fluconazole) at concentrations ranging from 0.4 to 1000 µg/mL. MHB and SDB media were added to a 96-well plate along with growth control groups for the microorganisms as the negative control group. Following the incubation period, the wells were incubated for 4 hours with 20 µL of resazurin (0.01%) dye. The color changes in the wells, which ranged from blue to pink, were used to interpret the results after 4 hours. The findings were averaged after the experiment was conducted three times.

3. FINDINGS

3.1. Antioxidant Activity and Total Phenolic Content

The antioxidant potential of *B. nigra* subsp. *anatolica* was evaluated, revealing promising results across different extracts. According to the findings, methanol, infusion, and ethyl acetate extracts were found to be effective, respectively. The most effective extract was found to be methanol with an IC₅₀ value of 0.516 mg/mL which is shown in Table 1. Consistent with previous studies, various extracts of *B. nigra* have demonstrated significant antioxidant capacities through different assays (ABTS, DPPH, FRAP, and CUPRAC) (Morteza-Semnani & Ghanbarimasir, 2019). In the ABTS radical scavenging assay, for instance, Ertaş *et al.* found that the aqueous extract of *B. nigra* subsp. *anatolica* at 100 µg/mL demonstrated 88% inhibition, outperforming common antioxidants like α-tocopherol and BHT at the same concentration (Ertaş *et al.*, 2014). Additionally, both the water and acetone extracts showed the highest antioxidant activities in the CUPRAC assay, indicating the versatility of different solvents in extracting potent antioxidant compounds from the plant. Similarly, Makowczyńska

et al. demonstrated the ability of methanolic extracts from *B. nigra* shoots (derived from in vitro explants, seeds, and wild-grown plants) to scavenge free radicals in the DPPH assay and reduce iron ions in the FRAP test (Makowczyńska *et al.*, 2015). These findings align with the results of the current study, where the *B. nigra* subsp. *anatolica* exhibited significant antioxidant activity. Additionally, the total phenol content of the extracts was found to be between 43.2 and 66.8 mg GAE/g ext and the results are given in Table 1. As per the findings, the *B. nigra* subsp. *anatolica* methanol extract has the most amount of total phenol (66.8 ± 0.01 mg GAE/g ext). The number of studies conducted on *B. nigra* subsp. *anatolica* is quite limited. In a study by Makowczyńska *et al.*, the total phenolic content of *B. nigra* methanol extracts was measured as 61.54 ± 0.75 and 93.41 ± 0.13 mg GAE/g ext. The results were quite similar to the total phenol amount of the methanol extract in this study. Furthermore, findings suggest that there is a positive relationship between the antioxidant activity of methanol extracts and their phenolic contents (Makowczyńska *et al.*, 2015).

Table 1. Total phenolic content (TPC) and DPPH[•] radical scavenging activity.

Extracts	TPC(mg GAE/g ext.)	DPPH (IC ₅₀) (mg/mL)
BA	43.2±0.02	0.738±0.02
BI	49.6±0.03	0.613±0.03
BM	66.8±0.01	0.516 ±0.02
GA	-	0.003±0.001

BA: Ethyl acetate extract; BI: Infusion extract; BM: Methanol extract, GA: Gallic acid; mg GAE/g extract: Total phenols expressed as gallic acid equal milligrams of gallic acid per gram (dry weight) of extract.

3.2. Antimicrobial Activity

The Minimum Inhibitory Concentration (MIC) values were determined using the broth microdilution method. Results from this investigation demonstrate that methanol and ethyl acetate extracts of *B. nigra* subsp. *anatolica* exhibit potent antimicrobial activity. The target under investigation and the extraction solvent can sometimes have a significant impact on a species' antimicrobial activity. The MIC values of methanol and ethyl acetate extracts varied between 97-3125 µg/mL (Table 2). The sensitivity of the standard microorganisms used in this study was determined against methanol and ethyl acetate extracts. The results suggest that the components in the ethyl acetate extract may be responsible for the antimicrobial activity. When a comparison is made between standard bacteria and yeast species based on MIC values, it can be interpreted that yeast species are more sensitive to plant extracts than bacteria species with low MIC values. The antifungal effects of methanol extracts against all tested fungal strains were very close to those of standard antifungals.

In the review published by Semnania and Ghanbarimasirb in 2019, it was emphasized that substances such as ballonigrin, dehydrohispanolone and hispanolone isolated from extracts of the *Ballota* genus showed very good antifungal and antibacterial activity (Morteza-Semnani & Ghanbarimasir, 2019). According to the results of the study conducted by Dulger and Dulger in 2012, methanol extracts of *B. acetabulosa* were 32 µg/mL against *Escherichia coli* and *Enterococcus faecalis* and 64 µg/mL against *C. albicans* (Dulger & Dulger, 2012). Compared to the results of our study, *B. nigra* subsp. *anatolica* methanolic extracts showed antifungal activity at a lower dose of 97 µg/mL and proved its potential as a more potent inhibitory agent. Dulger, Akı, and Dulger (2010) demonstrated that extracts of *B. nigra* subsp. *anatolica* possess both antibacterial and antifungal properties (Dulger *et al.*, 2010). Numerous factors, including non-specific heterogeneity in plants' production of secondary metabolites, could account for variations in antimicrobial activity among studies. Additionally, different assay techniques and extraction strategies may be used to recover active metabolites.

Table 2. Minimum inhibitory concentration (mg/mL).

Samples	<i>B. cereus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>L. monocytogenes</i>	<i>C. albicans</i>	<i>C. krusei</i>
BA	3.125	3.125	0.390	1.562	0.390	1.562
BI	-	-	-	-	-	-
BM	3.125	3.125	3.125	1.562	0.097	0.097
Erythromycin	0.062	0.125	0.250	0.125	-	-
Fluconazole	-	-	-	-	0.031	0.031

BA :Ethyl acetate extract; BI: Infusion extract; BM: Methanol extract; ; -: no activity

4. CONCLUSION

This study aims to shed light on the biological properties of *B. nigra* subsp. *anatolica*, a medicinal plant in Türkiye. In conclusion, the most researched bioactivities were those related to antimicrobial and antioxidant activity, which accounted for roughly 42.2% of the pharmacological research conducted on this genus. The results revealed the correlation between phenolic compounds and antioxidant activity. Additionally, methanol and ethyl acetate extracts of the plant showed significant antimicrobial activity in both standard bacteria and yeast. The outcome showed that methanolic extracts of *B. nigra* subsp. *anatolica* had a strong anti-yeast (antifungal) action. This activity could be a sign of the presence of plant chemicals or metabolic poisons. Therefore, more research should be done on these plant extracts since they may contain a novel substance that is useful against infections. To determine the primary active ingredients, more research is required to determine the primary active ingredients. To sum up, *B. nigra* subsp. *anatolica* merits consideration as a traditional medicinal plant with the potential for further development into a pharmaceutical herbal product.

Acknowledgments

The authors would like to thank Anadolu University, Faculty of Pharmacy. There is no project support.

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

Nagehan Saltan: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Zeynep Gülcan:** Methodology, Formal Analysis, and Writing -original draft, and Validation. **Pervin Soyer:** Methodology, Formal Analysis, and Writing -original draft, and Validation. Authors may edit this part based on their case.

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