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Determination of the antioxidant and antimicrobial activities and total phenolic content of *Borago officinalis* L. leaf extracts prepared by ultrasound-assisted extraction

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ABSTRACT: *Borago officinalis* L. is an edible wild and medicinal plant with reported antioxidant and antimicrobial properties; however, studies focusing on its leaves collected in Türkiye, particularly using ultrasound-assisted extraction, remain limited. Moreover, the effect of washing on the leaves before drying on their phytochemical composition and biological activities has not been sufficiently investigated. Therefore, this study aimed to evaluate comprehensively vthe antioxidant, total phenolic, and antimicrobial properties of *Borago officinalis* extracts prepared from washed and unwashed leaves using ultrasound-assisted extraction, while assessing whether washing influences the measured phytochemical and biological properties. Ethanolic, methanolic, and aqueous extracts were prepared from the leaves. Antioxidant activity was determined using the CUPRAC and ABTS assays, total phenolic content using the Folin–Ciocalteu method, and antimicrobial activity using agar well diffusion and broth microdilution methods. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) values were also determined. The total phenolic contents (mg GAE/g dw) of methanolic, ethanolic, and aqueous extracts were 475.22, 294.32, and 1039.87, respectively, for washed leaves, and 460.27, 188.06, and 1038.43, respectively, for unwashed leaves. CUPRAC values (mmol TE/g dw) were 1.465, 0.586, and 4.233 for washed leaves and 1.350, 0.298, and 3.207 for unwashed leaves, respectively, whereas ABTS values (mmol TE/g dw) were 6.547, 3.551, and 25.205 for washed leaves and 6.520, 4.371, and 22.473 for unwashed leaves, respectively. The ethanolic extract of unwashed leaves formed inhibition zones against *Acinetobacter baumannii* (7.18 mm) and *Escherichia coli* (8.59 mm). In the broth microdilution assay, the ethanolic extract of unwashed leaves and the ethanolic and methanolic extracts of washed leaves exhibited MIC values of 0.196–1.563 mg/mL against all tested microorganisms, whereas aqueous extracts showed MIC values of 0.098–>6.25 mg/mL (washed leaves) and 0.024–>6.25 mg/mL (unwashed leaves) together with MBC values 3.125->6.25 mg/mL (washed leaves) and 0.049–>6.25 mg/mL (unwashed leaves). Additionally among the all extracts the MFC values of washed leaves and unwashed leaves was 3.125>6.25 mg/mL. Overall, aqueous extracts exhibited the highest total phenolic contents and antioxidant capacities, while all extract types demonstrated antimicrobial activity against the tested microorganisms. The findings support the potential of *Borago officinalis* leaves as a natural source of phenolic and bioactive compounds and provide useful data for their potential applications in food, cosmetic, and phytotherapeutic fields.

KEYWORDS: *Borago officinalis* L.; antioxidant activity; phenolic content; antimicrobial activity; ultrasound-assisted extraction; UV-Vis spectroscopy.

1. INTRODUCTION

In order to use medicinal plants in the treatment of diseases, their quality, safety, and efficacy must be scientifically evaluated and documented. The ability of plant-derived antioxidants to protect cells against reactive oxygen species and other harmful agents has considerably increased interest in these natural products. Antioxidants are compounds capable of protecting cells from oxidative damage induced by free radicals. They exert their protective effects by scavenging and stabilizing free radicals, thereby reducing oxidative damage and contributing to the maintenance of cellular homeostasis. Antioxidants play an important role in preventing the oxidation of oxidizable compounds, thereby contributing to the reduction of

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the risk of bacterial infections, cancer, and cardiovascular diseases. Most of the oxygen utilized by cells is converted into water through enzymatic reactions. However, some enzymatic processes result in electron leakage to oxygen molecules, leading to the formation of reactive oxygen species and free radicals. In addition, exposure to ionizing radiation, motor vehicle exhaust emissions, carbon tetrachloride, and cigarette smoke can promote free radical generation. Excessive production of free radicals causes oxidative stress, which has been implicated in the pathogenesis of numerous diseases, including periodontitis, cancer, inflammatory disorders, cardiovascular and respiratory diseases, diabetes mellitus, male infertility, neurodegenerative disorders, nephropathy, and the aging process [1].

Antimicrobial resistance is one of the most urgent global public health threats of the twenty-first century. It is primarily driven by the widespread and inappropriate use of antimicrobial agents in human medicine, veterinary practice, and agriculture. As bacteria evolve mechanisms that enable them to survive exposure to antibiotics, conventional treatments become less effective, making common infections increasingly difficult to treat and significantly increasing the risk of severe illness, complications, and mortality [2, 3]. In addition, the emergence of resistance to antifungal agents has further complicated the treatment of candidiasis and other fungal infections [4]. Addressing this growing challenge requires a multifaceted approach, including the rational use of antimicrobial agents, improved hygiene and infection control practices, and continued investment in the research and development of novel therapeutic strategies. Medicinal plants have been used for centuries in the treatment of infectious diseases and represent a rich source of biologically active compounds with antimicrobial potential. Consequently, increasing attention has been directed toward medicinal plants as promising sources of novel antimicrobial agents that may serve as complementary therapies to conventional antibiotics in the management of infectious diseases.

Borago officinalis L. (*B. officinalis*) (hodan), is an annual herbaceous plant belonging to the Boraginaceae family. It is believed to be native to Anatolia and Syria and is widely distributed throughout South America, North Africa, and the Mediterranean region. In Türkiye, it occurs naturally in several regions, particularly in Western and Northern Anatolia. Owing to its attractive blue and pink flowers, *B. officinalis* is cultivated as an ornamental plant in some areas, although it also grows spontaneously along roadsides and in agricultural fields. Due to its rich phytochemical composition, *B. officinalis* has been widely used since ancient times both as an edible wild plant and in traditional and complementary medicine. Its leaves are consumed as a vegetable, eaten fresh in salads, or preserved as pickles. Traditionally, the plant has been used to alleviate swelling and inflammation, respiratory disorders, melancholy, and pain associated with injured or irritated tissues. In addition, numerous *in vitro* and *in vivo* studies have demonstrated a broad spectrum of biological and pharmacological activities, including antiinflammatory, antioxidant, antimicrobial, anticancer, antigenotoxic, anti-obesity, antidepressant, diuretic, emollient, laxative, antipyretic, antianemic, and menstrual analgesic effects. These biological activities are largely attributed to the diverse bioactive constituents present in different parts of the plant, including the leaves, flowers, seeds, and roots, such as phenolic acids, flavonoids, anthocyanins, alkaloids, and terpenes. Furthermore, the seed oil, which is particularly rich in γ-linolenic acid, is regarded as a valuable natural source in complementary medicine because of its potential to regulate hormonal function and its antihypertensive properties [5, 6, 7, 8, 9].

One of the major classes of bioactive compounds present in *B. officinalis* is polyphenols, which are well known for their free radical scavenging properties. The antioxidant activity of phenolic compounds, including phenolic acids, flavonoids, and their derivatives, is primarily attributed to the presence of hydroxyl groups attached to aromatic rings [10]. Previous studies have suggested that variations in the antioxidant capacity of *B. officinalis* may be partly associated with the type of extract used, as well as qualitative and quantitative differences in its phenolic composition [11]. The literature includes numerous studies investigating the total phenolic content and antioxidant activity of extracts obtained from different parts of *B. officinalis* (roots, stems, leaves, and predominantly seeds) using various solvents, mainly ethanol (EtOH), methanol (MeOH) and aqueous (A), and conventional extraction methods such as infusion and maceration. In these studies, antioxidant activity has been evaluated predominantly by the DPPH free radical scavenging assay and expressed as the half-maximal inhibitory concentration (IC_{50}), whereas only a limited number of studies have employed the ABTS assay.

Studies investigating the antioxidant activity and total phenolic content of *B. officinalis* leaves have been conducted using different extraction methods, solvents, and antioxidant assays. Zemmouri et al. (2014) reported that EtOH and A leaf extracts obtained by maceration and decoction from plants collected in Algeria exhibited strong antioxidant activity and high phenolic contents, as determined by the DPPH, nitroblue tetrazolium, and phosphomolybdenum assays [11]. In Spain, de Ciriano et al. (2009) evaluated the antioxidant

capacity of lyophilized A leaf extracts using the DPPH and ABTS assays and determined the total phenolic content. They suggested that the extracts could serve as effective natural antioxidants for controlling lipid oxidation in ω-3 polyunsaturated fatty acid-enriched dry fermented sausages [12]. Segovia et al. (2014) optimized the extraction of polyphenols from A and EtOH leaf extracts using response surface methodology and evaluated their ORAC for potential application in emulsion systems [13]. García-Herreros et al. (2010) investigated the antioxidant activity and total phenolic content of A leaf extracts using the DPPH and ABTS assays [14]. More recently, Tejedor-Calvo et al. (2026) compared conventional and ultrasound-assisted extraction using different hydroethanolic mixtures and evaluated the resulting extracts in terms of total phenolic content and antioxidant activity determined by the DPPH and ABTS assays to establish optimal extraction conditions [15]. Pereira et al. (2011) reported high antioxidant activity and phenolic contents in MeOH leaf extracts from Portugal based on the DPPH radical scavenging assay and the β-carotene bleaching test [16]. In Türkiye, Hasdemir et al. (2023) investigated the applicability of *B. officinalis* leaf essential oil for preventing the oxidation of fish oil intended for the food industry and reported relatively weak DPPH radical scavenging activity [17]. Mhamdi et al. (2010) compared the total phenolic contents (TPC) and IC_{50} values of leaves collected during different growth stages in Tunisia and concluded that antioxidant activity was not directly correlated with total phenolic content [18]. In Italy, Conforti et al. (2008) evaluated hydroalcoholic leaf extracts using the DPPH assay, lipid peroxidation inhibition, β-carotene bleaching assay, and the Folin–Ciocalteu method [19]. MeOH leaf extracts have also been evaluated by Rodríguez-Magaña et al. (2019) in Mexico using Soxhlet extraction and the DPPH assay [20], by Jaradat et al. (2016) in Palestine using the DPPH assay [21], and by Abu-Qaoud et al. (2018) in Palestine for total phenolic content determination [22]. Singh et al. (2014) investigated MeOH, chloroform, and petroleum ether leaf extracts in India using the DPPH and nitric oxide radical scavenging assays together with total phenolic content analysis [23]. Additional studies have evaluated MeOH leaf extracts by the DPPH assay in Lithuania [24], hydroacetonic extracts using the DPPH assay and the Folin–Ciocalteu method in Lebanon [25], A extracts using the DPPH and FRAP assays together with total phenolic content analysis in Poland [26], EtOH extracts for phenolic content in Iraq [27], and MeOH extracts for phenolic content in Iran [28].

Previous studies on the antimicrobial activity of *B. officinalis* have reported varying degrees of activity against a range of bacterial and fungal microorganisms. Kadhum et al. (2009) reported that *B. officinalis* extracts were effective against clinical strains of *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermisis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), and *Klebsiella pneumoniae* (*K. pneumoniae*) based on the disk diffusion method [2]. Similarly, Monari et al. (2025) reported that the *B. officinalis* samples investigated exhibited consistently greater activity against the Gram-negative bacterium *E. coli*. The lowest minimum inhibitory concentration (MIC) values against *E. coli* were 0.47 mg/mL for flower decoctions and 0.94 mg/mL for leaf infusions of *B. officinalis* [3]. Al-Qurashi et al. (2019) found that EtOH extracts of *B. officinalis* (0.4 g/1.5 mL) were effective against *P. aeruginosa*, by forming an 8-mm inhibition zone, but were ineffective against *Klebsiella* spp [29]. Barbakadze et al. (2023) investigated the antimicrobial activity of *B. officinalis* root extracts and reported MIC/MBC values of 1.50/3.00 mg/mL for *S. aureus*, 1.00/1.50 mg/mL for *E. coli*, 1.00/1.50 mg/mL for *P. aeruginosa*, and 0.37 mg/mL for *Candida albicans* (*C. albicans*) [30]. Eidi et al. (2014) reported MIC and minimum bactericidal concentration (MBC) values of 3.125 and 6.25 mg/mL, respectively, for *C. albicans* treated with hydroalcoholic extracts of *B. officinalis* [4].

The aim of this study was to comprehensively evaluate the antioxidant activities, total phenolic contents, and antimicrobial properties of EtOH, MeOH, and A extracts obtained from *B. officinalis* leaves collected in Zonguldak, Türkiye, using ultrasound-assisted extraction. Although previous studies have investigated the phenolic content, antioxidant activity, and antimicrobial properties of extracts obtained from different parts of *B. officinalis*, studies specifically focusing on the ultrasound-assisted extraction of its leaves appear to be limited. Furthermore, although the ABTS assay has been used to evaluate the antioxidant activity of *B. officinalis* extracts, studies reporting ABTS results in mmol TE/g dw are limited, with results in many previous studies being expressed as IC_{50} values. The use of the CUPRAC assay for evaluating the antioxidant capacity of *B. officinalis* leaf extracts also appears to be limited. In addition, studies simultaneously investigating the antioxidant, total phenolic, and antimicrobial properties of *B. officinalis* leaves collected in Türkiye remain scarce. Accordingly, the total phenolic contents, antioxidant activities determined by the CUPRAC and ABTS assays, and antimicrobial properties of EtOH, MeOH, and A extracts prepared from washed and unwashed leaves using ultrasound-assisted extraction were evaluated. To assess the effect of leaf washing, a portion of the collected leaves was washed with distilled water, while the remaining leaves were dried without washing, and extracts were prepared separately from the two sample groups. The findings were

then compared with previously reported data for *B. officinalis* grown in different geographical regions. This approach was designed to provide additional data on the effects of sample preparation conditions and geographical variation on the phenolic content, antioxidant properties, and antimicrobial activity of *B. officinalis* leaves and to contribute to the existing literature on the *in vitro* biological properties of this plant.

2. RESULTS AND DISCUSSION

2.1. Total phenolic content of *B. officinalis* extracts

The TPC values of the extracts are presented in Table 1 and Fig. 1. The highest TPC was observed in the A extract of the washed *B. officinalis* leaves, with a value of 1039.87 ± 15.09 mg GAE/g dw. As shown in Table 1, the A extract prepared from the unwashed leaves exhibited a comparable TPC (1038.43 ± 10.90 mg GAE/g dw). In contrast, the lowest TPC was determined in the EtOH extract of the unwashed leaves (188.06 ± 2.53 mg GAE/g dw), whereas the corresponding EtOH extract of the washed leaves contained a considerably higher phenolic content (294.32 ± 5.39 mg GAE/g dw).

Prior to the Folin–Ciocalteu assay, the A extracts were diluted five-fold with distilled water to ensure that their absorbance values fell within the linear range of the gallic acid calibration curve. The dilution factor was taken into account when calculating the TPC.

Table 1. Total phenolic contents and antioxidant properties of *B. officinalis* leaf extracts prepared from washed and unwashed leaves

Sample	Solvent	TPC (mg GAE/g dw)	CUPRAC (mmol TE/g dw)	ABTS (mmol TE/g dw)
WL	MeOH	475.22±4.75^{b}	1.465±0.023^{c}	6.547±0.110^{c}
UL	MeOH	460.27±4.31^{b}	1.350±0.026^{d}	6.520±0.070^{c}
WL	EtOH	294.32±5.39^{c}	0.586±0.008^{e}	3.551±0.054^{e}
UL	EtOH	188.06±2.53^{d}	0.298±0.005^{f}	4.371±0.054^{d}
WL	A	1039.87±15.09^{a}	4.233±0.025^{a}	25.205±0.137^{a}
UL	A	1038.43±10.90^{a}	3.207±0.040^{b}	22.473±0.220^{b}

Values are expressed as mean ± SD (n = 3 independent extraction replicates). Each extract was analyzed in triplicate. Different letters within the same column indicate significant differences ($p < 0.05$).
WL: Washed leaves (leaves were washed with distilled water prior to drying and extraction).
UL: Unwashed leaves.
MeOH: Methanol.
EtOH: Ethanol.
A: Aqueous.
GAE: Gallic acid equivalents.
TE: Trolox equivalents.

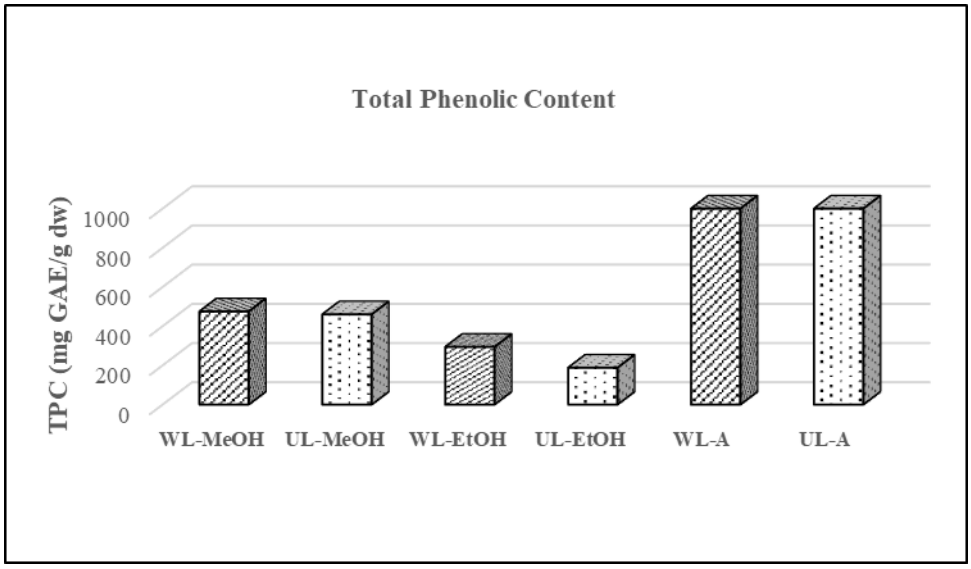

Figure 1. Total phenolic content (TPC) of *B. officinalis* leaves collected from Zonguldak, Türkiye, extracted with ethanol (EtOH), methanol (MeOH), and aqueous (A) from washed (WL) and unwashed (UL) samples. Results are expressed as mg gallic acid equivalents per g dry weight (mg GAE/g dw).

In a study by Zemmouri et al. (2014), the TPC of the A extract obtained by decoction and the EtOH extract obtained by maceration of *B. officinalis* leaves were reported as 35.48 ± 2.70 and 94.09 ± 1.72 mg GAE/g dw, respectively [11]. In another study, the TPC of the lyophilized A extract was reported as 57.7 mg GAE/g extract, corresponding to approximately 0.9 mg GAE/g fresh plant material [12]. Pereira et al. (2011) reported a TPC of 113.58 ± 0.92 mg GAE/g extract for MeOH extracts [16]. Similarly, TPC values of 27.05 mg GAE/g dw for EtOH extracts and 41.84 ± 0.20 mg GAE/g dw for A extracts were reported by Segovia et al. (2014) [13]. For *B. officinalis* collected in Tunisia, TPC values of MeOH extracts were reported as 3.60 mg GAE/g dw in young leaves and 4.80 mg GAE/g dw in mature leaves [18]. Several other studies have reported TPC values of 97 mg/g for hydroethanolic extracts [19], 298.607 mg GAE/100 g dw for hydroacetonic extracts [25], 2.36 mg GAE/g fw for A leaf extracts [14], 88.8 mg GAE/g extract for EtOH extracts [27], 5.21 mg GAE/g for MeOH extracts [22], 14.89–17.44 mg GAE/g for A extracts [26], and 38 ± 0.45 mg GAE/g for MeOH extracts [28].

Compared with previous studies using similar extraction solvents and reporting TPC on a comparable basis, the present study yielded higher TPC values. The higher TPC observed in the present study may be associated with the use of ultrasound-assisted extraction for *B. officinalis* leaves, as well as with differences in the geographical origin of the plant material collected in Türkiye. Since ultrasound-assisted extraction has been less frequently employed for *B. officinalis* leaves in previous studies and studies on *B. officinalis* leaves collected in Türkiye are also limited, these factors may have contributed to the higher phenolic contents observed in the present study. The significant washing × solvent interaction ($p < 0.001$) indicated that the effect of washing on TPC depended on the extraction solvent. According to the Tukey post hoc test, washing resulted in a significant difference in TPC for the EtOH extracts, whereas no significant differences were observed between the washed and unwashed MeOH or A extracts.

2.2. Antioxidant capacity determined by the CUPRAC assay

The CUPRAC antioxidant capacities of the extracts are presented in Table 1 and Fig. 2. According to the CUPRAC assay, the highest antioxidant capacity was observed for the A extract of the washed *B. officinalis* leaves, with a value of 4.233 ± 0.025 mmol TE/g dw, whereas the lowest value was determined for the EtOH extract of the unwashed leaves (0.298 ± 0.005 mmol TE/g dw). As shown in Table 1, the CUPRAC antioxidant capacity values varied among the extracts prepared with the three solvents from both washed and unwashed leaves.

Prior to the CUPRAC assay, the EtOH, MeOH, and A extracts were diluted with distilled water to ensure that their absorbance values fell within the linear range of the Trolox calibration curve. The dilution factor was taken into account when calculating the antioxidant capacity values.

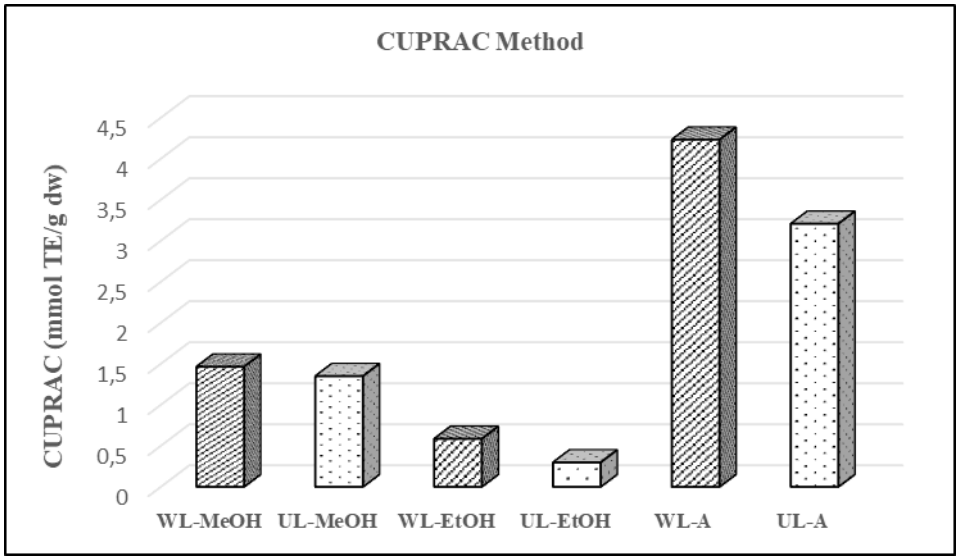

Figure 2. CUPRAC antioxidant capacity of *B. officinalis* leaves collected from Zonguldak, Türkiye, extracted with ethanol (EtOH), methanol (MeOH), and aqueous (A) from washed (WL) and unwashed (UL) samples. Results are expressed as mmol Trolox equivalents per g dry weight (mmol TE/g dw).

The CUPRAC antioxidant capacity values obtained in the present study were calculated from the Trolox calibration curve and expressed as mmol Trolox equivalents per gram dry weight (mmol TE/g dw). This unit was used consistently for the CUPRAC results throughout the text, tables, and figures. According to the Tukey post hoc test, significant differences between washed and unwashed extracts were observed for all three extraction solvents. The use of the CUPRAC assay for evaluating the antioxidant capacity of *B. officinalis* leaf extracts appears to be limited in the literature; therefore, the present results could not be directly compared with previous studies.

2.3. Antioxidant activity determined by the ABTS assay

The ABTS radical cation scavenging activities of the extracts are presented in Table 1 and Fig. 3. According to the ABTS assay, the highest antioxidant activity was observed for the A extract of the washed *B. officinalis* leaves, with a value of 25.205 ± 0.137 mmol TE/g dw, whereas the lowest value was determined for the EtOH extract of the washed leaves (3.551 ± 0.054 mmol TE/g dw). As shown in Table 1, the ABTS radical cation scavenging activities of the MeOH extracts prepared from washed and unwashed leaves were comparable, whereas differences were observed between the EtOH and A extracts obtained from washed and unwashed leaves.

Prior to the ABTS assay, the EtOH, MeOH, and A extracts were diluted with distilled water to ensure that their absorbance values fell within the linear range of the Trolox calibration curve. The dilution factor was taken into account in the calculation of antioxidant activity.

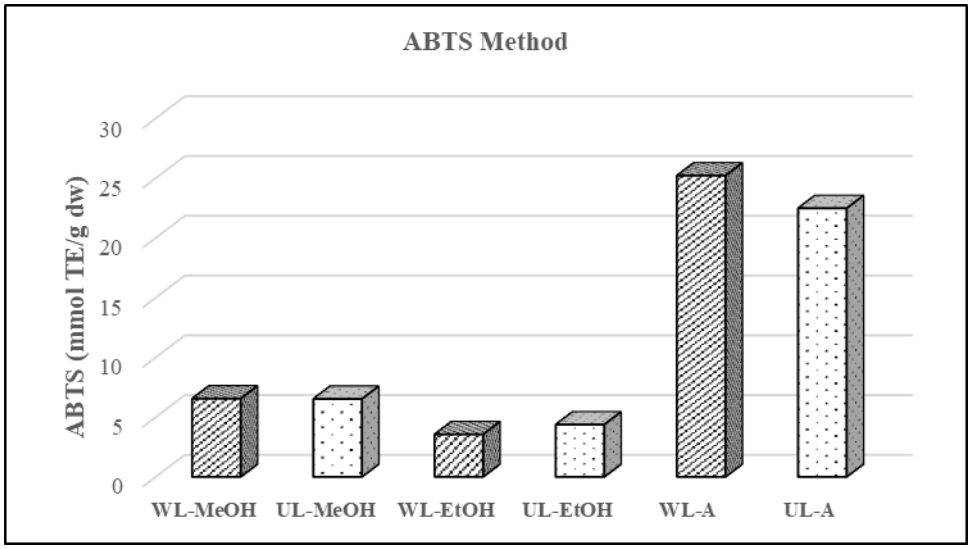

Figure 3. ABTS antioxidant activity of *B. officinalis* leaves collected from Zonguldak, Türkiye, obtained using ethanol (EtOH), methanol (MeOH), and aqueous (A) extracts from washed (WL) and unwashed (UL) samples. Results are expressed as mmol Trolox equivalents per g dry weight (mmol TE/g dw).

According to the Tukey post hoc test, significant differences between washed and unwashed extracts were observed for the EtOH and A extracts, whereas no significant difference was observed for the MeOH extracts. Regarding previous studies on *B. officinalis* leaves, ABTS antioxidant activity has been reported as 50.5 mg TE/g extract for lyophilized aqueous extracts [12], 117 µmol/g extract for hydroethanolic extracts [15], and 1128.38 µg TE/g fresh weight for aqueous extracts of fresh leaves [14]. These values were not converted to mmol TE/g dw because they were reported on different sample bases and in different units, and the information required for a reliable conversion to a common dry-weight basis was not available. Therefore, direct quantitative comparisons with the present mmol TE/g dw results were avoided. Similarly, ABTS results reported as IC_{50} values were not directly compared with the present results because IC_{50} represents a different expression and calculation approach.

A significant washing × solvent interaction was observed for TPC, CUPRAC, and ABTS values ($p < 0.001$), indicating that the effect of washing on total phenolic content and antioxidant properties was solvent-dependent, with the differences between washed and unwashed extracts varying according to the extraction solvent.

2.4. Antimicrobial activity of *B. officinalis* extracts

The antimicrobial activities of *B. officinalis* extracts against *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228), *P. aeruginosa* (ATCC 27853), *Acinetobacter baumannii* (*A. baumannii*) (ATCC 19606), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 4352), and the yeast *C. albicans* (ATCC 90028) were tested.

As shown in Table 2, the EtOH extract of *B. officinalis* leaves (washed leaves) exhibited the strongest activity against *A. baumannii* and *K. pneumoniae*, with MIC values of 0.196 and 0.390 mg/mL and MBC values of 0.781 and 1.563 mg/mL, respectively. The MeOH extract of washed *B. officinalis* leaves showed the strongest activity against *A. baumannii*, with an MIC value of 0.196 mg/mL and an MBC value of 1.563 mg/mL. Similarly, the A extract of washed *B. officinalis* leaves exhibited the strongest activity against *P. aeruginosa*, with an MIC value of 0.196 mg/mL and an MBC value of 3.125 mg/mL; however, it showed no activity against *C. albicans*.

Table 2. Antimicrobial activity of *B. officinalis* leaf extracts prepared from washed leaves

Microorganism	Ethanol Extract			Methanol Extract			Aqueous Extract		
	ZD (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)	ZD (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)	ZD (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)
***S. aureus* ATCC 29213**	0	0.390	3.125	0	0.196	6.25	0	0.098	>6.25
***S. epidermidis* ATCC 12228**	0	0.390	3.125	0	0.390	1.563	0	0.196	>6.25
***P. aeruginosa* ATCC 27853**	0	0.196	3.125	0	0.196	3.125	0	0.196	3.125
***A. baumannii* ATCC 19606**	0	0.196	0.781	0	0.196	1.563	0	0.390	3.125
***E. coli* ATCC 25922**	0	0.781	1.563	0	0.781	3.125	0	1.563	6.25
***K. pneumoniae* ATCC 4352**	0	0.390	1.563	0	0.390	>6.25	0	0.781	>6.25
***C. albicans* ATCC 90028**	0	1.563	>6.25	0	1.563	3.125	0	>6.25	>6.25

ZD: Zone diameter.
MIC: Minimum Inhibitory Concentration.
MBC: Minimum Bactericidal Concentration.
MFC: Minimum Fungicidal Concentration.

As shown in Table 3, the EtOH extract of *B. officinalis* prepared from unwashed leaves exhibited clear inhibition zones against *A. baumannii* (7.18 mm) and *E. coli* (8.59 mm). In the broth microdilution assay, the EtOH extract of unwashed leaves showed the strongest activity against *K. pneumoniae* (MIC: 0.390 mg/mL; MBC: 1.563 mg/mL), followed by *S. epidermidis* and *E. coli*, which exhibited the same MIC and MBC values (MIC: 0.390 mg/mL; MBC: 1.563 mg/mL). The MeOH extract of *B. officinalis* prepared from unwashed leaves showed the strongest activity against *K. pneumoniae*, with an MIC of 0.049 mg/mL and an MBC of 0.098 mg/mL. The A extract of *B. officinalis* prepared from unwashed leaves showed the strongest activity against *S. aureus* (MIC: 0.024 mg/mL; MBC: 0.049 mg/mL), followed by *S. epidermidis* and *K. pneumoniae*, which exhibited the same MIC value (0.098 mg/mL) and MBC value (0.781 mg/mL). Among the three extracts, only the EtOH extract of *B. officinalis* prepared from unwashed leaves exhibited a fungistatic effect against *C. albicans*, with an MIC of 1.563 mg/mL.

Table 3. Antimicrobial activity of *B. officinalis* leaf extracts prepared from unwashed leaves

Microorganism	Ethanol Extract			Methanol Extract			Aqueous Extract		
	ZD (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)	ZD (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)	ZD (mm)	MIC (mg/mL)	MBC/MFC (mg/mL)
***S. aureus* ATCC 29213**	0	0.781	3.125	0	0.390	0.781	0	0.024	0.049
***S. epidermidis* ATCC 12228**	0	0.390	1.563	0	0.390	1.563	0	0.098	1.563
***P. aeruginosa* ATCC 27853**	0	0.196	3.125	0	0.781	0.781	0	0.196	0.781
***A. baumannii* ATCC 19606**	7.18±0.34	0.781	3.125	0	0.781	0.781	0	0.781	6.25
***E. coli* ATCC 25922**	8.59±0.27	0.390	1.563	0	0.781	>6.25	0	1.563	>6.25
***K. pneumoniae* ATCC 4352**	0	0.390	0.781	0	0.049	0.098	0	0.098	1.563
***C. albicans* ATCC 90028**	0	1.563	>6.25	0	>6.25	>6.25	0	>6.25	>6.25

ZD: Zone diameter.
MIC: Minimum Inhibitory Concentration.
MBC: Minimum Bactericidal Concentration.
MFC: Minimum Fungicidal Concentration.

In the present study, several extracts showed no zone of inhibition (ZD = 0 mm) but exhibited measurable MIC values. This contrast highlights the inherent limitations of the agar diffusion method for certain plant extracts. The agar matrix may act as a physical barrier that restricts the diffusion of large molecules or non-polar and hydrophobic constituents. Consequently, potent antimicrobial compounds may fail to produce a visible zone of inhibition if they cannot adequately diffuse from the well into the surrounding agar medium. In contrast, the broth microdilution method minimizes diffusion-related limitations by maintaining the test compounds in direct contact with the microorganisms in a liquid medium. These findings indicate that a ZD of 0 mm does not necessarily imply an absence of antimicrobial activity and emphasize the importance of using MIC assays to complement agar diffusion-based screening. Therefore, for the specific extract types evaluated in the present study, the broth microdilution method provided a more sensitive assessment of antimicrobial activity than the agar diffusion method.

3. CONCLUSION

The present study showed that *B. officinalis* L. leaves collected in Zonguldak, Türkiye, had measurable total phenolic content and exhibited antioxidant and antimicrobial activities. Among the extracts evaluated, the A extracts exhibited the highest TPC as well as the greatest antioxidant capacities according to the CUPRAC and ABTS assays. The MeOH extracts generally showed higher phenolic contents and antioxidant capacities than the EtOH extracts. These findings represent *in vitro* measurements and should not be interpreted as evidence of direct dietary, food, cosmetic, or phytotherapeutic efficacy.

The washing process affected the phenolic content and antioxidant properties in a solvent-dependent manner. For TPC, washing resulted in a significant difference only for the EtOH extracts, whereas no significant differences were observed between the washed and unwashed MeOH or aqueous extracts. For CUPRAC, significant differences between washed and unwashed extracts were observed for all three extraction solvents, whereas for ABTS, significant differences were observed for the EtOH and aqueous extracts but not for the MeOH extracts. The antimicrobial activity profiles also varied among the extracts prepared from washed and unwashed leaves, with differences observed in the microorganisms showing the highest susceptibility to the respective extracts. Minor differences were observed between the washed and unwashed MeOH extracts, which exhibited the strongest activity against *A. baumannii* and *K. pneumoniae*, respectively. More pronounced differences were observed for the EtOH extracts, with the washed-leaf extract showing the strongest activity against *A. baumannii* and *K. pneumoniae*, whereas the unwashed-leaf extract showed the strongest activity against *K. pneumoniae*, *S. epidermidis*, and *E. coli*. Similarly, differences were observed for the A extracts, with the washed-leaf extract showing the strongest activity against *P. aeruginosa*,

whereas the unwashed-leaf extract showed the strongest activity against *S. aureus*, *S. epidermidis*, and *K. pneumoniae*. All three extract types (EtOH, MeOH, and A) exhibited antibacterial activity against the tested bacterial strains, while the EtOH extract of *B. officinalis* also exhibited activity against *C. albicans*.

To the best of our knowledge, few studies have investigated *B. officinalis* using ultrasound-assisted extraction, and the present study provides data on its antioxidant capacity as determined by the CUPRAC assay. Furthermore, reporting antioxidant results as mmol TE/g dry weight provides quantitative data in an expression format that is relatively scarce in the existing literature, particularly for ABTS assay results. This study also contributes to the limited body of literature on the antioxidant activity and phenolic content of *B. officinalis* leaves collected in Türkiye.

The higher phenolic contents and antioxidant capacities obtained in the present study compared with those reported for similar solvents in previous studies may be associated with the use of ultrasound-assisted extraction, differences in sample preparation conditions, and geographical variation. Overall, the findings provide preliminary *in vitro* evidence of the total phenolic content, antioxidant properties, and antimicrobial activity of *B. officinalis* leaves. Further studies, including investigations of safety, bioavailability, stability, and biological effects under appropriate experimental conditions, are required before any potential practical applications can be assessed.

4. MATERIALS AND METHODS

4.1. Chemicals

The following analytical grade chemicals were used in this study: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), neocuproine (2,9-dimethyl-1,10- phenanthroline), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate ($K_2S_2O_8$), sodium carbonate (Na_2CO_3), gallic acid ($C_7H_6O_5$), methanol (99.9%), ethanol (99%), copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), ammonium acetate (CH_3COONH_4), and acetic acid (CH_3COOH), all purchased from Merck (Darmstadt, Germany). Folin–Ciocalteu reagent (FCR) [$3H_2O \cdot P_2O_5 \cdot 13WO_3 \cdot 5MoO_3 \cdot 10H_2O$] was obtained from Sigma-Aldrich (Steinheim, Germany). All chemicals and reagents used were of analytical grade.

4.2. Instrumentation

The instruments used in this study included a UV–Vis spectrophotometer (Shimadzu UV-2550, Japan), a laboratory oven (Memmert UM 400, Germany), an ultrapure water purification system (NUVE ND 4, Türkiye), an analytical balance (Kern ABJ 220-4NM, Germany), an ultrasonic bath (Bandelin Sonorex, Germany), a vortex mixer (IKA MS2 Minishaker, Germany), a mechanical grinder, and an autoclave (Hirayama HV-85L, Japan).

4.3. Plant material and extraction

B. officinalis L. samples were collected from Zonguldak, Türkiye, during July–August 2022. A portion of the collected plant material was washed with distilled water, while the remaining material was left unwashed. Both washed and unwashed samples were then dried at room temperature in the shade, protected from direct sunlight. The dried samples were stored under appropriate conditions until further analysis. Botanical identification was performed by Dr. İsmail Şenkardeş from the Department of Pharmaceutical Botany, Faculty of Pharmacy, Marmara University. Following identification, the leaves were separated from the other plant parts and ground into a homogeneous fine powder using a mechanical grinder. The powdered samples were stored at room temperature in light-protected amber glass bottles until analysis.

The extraction procedure was carried out according to the method described by Yılmaz et al. (2019), with minor modifications [31]. Briefly, 0.5 g of powdered leaf sample was transferred into a stoppered Erlenmeyer flask, and 20 mL of extraction solvent was added, corresponding to a solvent-to-solid ratio of 40 mL/g per extraction step. Ultrasound-assisted extraction was performed using a Bandelin Sonorex Super RK 100 H ultrasonic bath (Bandelin, Germany) at a frequency of 35 kHz and a power range of 80–320 W, at 40 °C for 1 h. The extract was subsequently filtered through Macherey-Nagel Blue Ribbon filter paper (Macherey-Nagel, Germany), after which the plant residue was re-extracted with an additional 20 mL of the same solvent under identical conditions. The same extraction procedure was applied using EtOH, MeOH and distilled water as extraction solvents. The resulting extracts were filtered and stored in amber glass bottles at 4 °C until analysis. For each extraction solvent and sample type, three independent extractions were performed using separate 0.5-g portions of plant material under identical conditions, resulting in three independent extracts (n

= 3). For the determination of total phenolic content, each independent extract was analyzed in triplicate, and the mean of the three analytical measurements was calculated for each extract. Final results were expressed as mean ± standard deviation (SD) based on the three independent extraction replicates (n = 3). All analytical results were expressed on a dry weight (dw) basis.

4.4. Antioxidant Activity

4.4.1. Determination of ABTS radical cation scavenging activity

ABTS radical scavenging activity was determined using a modified method based on the procedures reported by Re et al. (1999) and Rice-Evans and Miller (1994) [32, 33]. The ABTS solution was prepared at a concentration of 7.0 mM, and $K_2S_2O_8$ was added to achieve a final persulfate concentration of 2.45 mM. The mixture was incubated at room temperature in the dark for 16 h to allow the formation of the $ABTS^{\bullet+}$ radical cation. The resulting $ABTS^{\bullet+}$ solution was diluted 1:50 (v/v) with EtOH and adjusted to an absorbance of approximately 0.700 at 734 nm. A calibration curve was prepared using Trolox standard solutions at different concentrations prepared from a 1 mM Trolox stock solution. For the assay, 2.00 mL of the $ABTS^{\bullet+}$ solution was mixed with an appropriate volume of Trolox standard solution, and the final volume was adjusted to 3.00 mL with EtOH. The mixtures were vortexed and incubated at room temperature in the dark for 6 min, after which the absorbance was measured at 734 nm. A mixture of 2.00 mL of $ABTS^{\bullet+}$ solution and 1.00 mL of EtOH was used as the reference solution. ABTS radical cation scavenging activity was calculated using the following equation:

$$\text{ABTS radical cation scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 represents the absorbance of the reference solution and A_1 represents the absorbance of the Trolox standard or plant extract. The absorbance values obtained for the Trolox standard solutions were used to construct a calibration curve by plotting absorbance against Trolox concentration. The antioxidant activities of the plant extracts were determined under the same conditions, and their Trolox equivalent values were calculated from the calibration curve. The results were expressed as mmol Trolox equivalents (TE)/g dry weight (dw). The dilution factor was taken into account in the calculation. Each independent extract was analyzed in triplicate, and the mean of the analytical measurements was used for statistical analysis.

4.4.2. Determination of CUPRAC antioxidant capacity

The CUPRAC assay was performed according to the procedure reported by Apak et al. (2004) [34]. The reaction mixture consisted of 1.00 mL $CuCl_2$ solution, 1.00 mL neocuproine (Nc) solution, 1.00 mL CH_3COONH_4 buffer, an appropriate volume of Trolox standard solution or plant extract, and distilled water to a final volume. After vortexing, the mixtures were incubated at room temperature in the dark for 30 min to allow the formation of the Cu(I)-Nc chelate through the reduction of the Cu(II)-Nc complex by antioxidant compounds. Absorbance was measured at 450 nm against the reference solution. A calibration curve was constructed using Trolox standard solutions at different concentrations by plotting absorbance against Trolox concentration. The antioxidant capacities of the plant extracts were determined under the same conditions and calculated from the Trolox calibration curve. The results were expressed as mmol Trolox equivalents (TE)/g dry weight (dw). Prior to the CUPRAC assay, the extracts were diluted with distilled water to ensure that their absorbance values fell within the linear range of the Trolox calibration curve, and the dilution factor was taken into account in the calculation. Each independent extract was analyzed in triplicate, and the mean of the analytical measurements was used for statistical analysis.

4.4.3. Determination of total phenolic content

TPC was determined using the Folin–Ciocalteu method according to Albayrak et al. (2010) and Magalhães et al. (2008) [35, 36]. The Folin–Ciocalteu reagent was diluted 1:3 (v/v) with distilled water before use. A calibration curve was prepared using gallic acid standard solutions at different concentrations prepared from a stock solution in MeOH. For the assay, an appropriate volume of gallic acid standard solution or plant extract was mixed with distilled water and 0.100 mL of the diluted Folin–Ciocalteu reagent. After 3 min, 3.00 mL of 2% (w/v) Na_2CO_3 solution was added, and the reaction mixture was incubated at room temperature in the dark for 2 h. Absorbance was measured at 760 nm against the reference solution. The gallic acid calibration curve was constructed by plotting absorbance against gallic acid concentration. TPC was calculated from the calibration curve and expressed as mg gallic acid equivalents (GAE)/g dry weight (dw). For the aqueous extracts, the dilution factor applied before the assay was taken into account in the calculation. Each

independent extract was analyzed in triplicate, and the mean of the analytical measurements was used for statistical analysis.

4.5. Antimicrobial Activity

The antimicrobial activities of *B. officinalis* extracts were evaluated using the agar well diffusion and broth microdilution methods. The MIC, MBC, and MFC values were determined according to standard microbiological procedures.

4.5.1. Microorganisms

The test microorganisms included *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228), *P. aeruginosa* (ATCC 27853), *A. baumannii* (ATCC 19606), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 4352), and the yeast *C. albicans* (ATCC 90028).

4.5.2. Determination of Agar Well Diffusion Method

The antimicrobial activity of the *B. officinalis* extracts was initially evaluated using the agar well diffusion method. Bacterial suspensions were prepared from colonies grown on tryptic soy agar (TSA) at 37 °C for 24 h, whereas the yeast suspension was prepared from colonies grown on Sabouraud dextrose agar (SDA) at 37 °C for 48 h. Bacterial suspensions were adjusted to approximately 1×10^8 CFU/mL and the yeast suspension to 1×10^6 CFU/mL using the 0.5 McFarland turbidity standard. The standardized suspensions were spread onto Mueller–Hinton agar (MHA) plates for bacteria and SDA plates for yeast using sterile cotton swabs. Wells (5 mm in diameter) were punched into the agar using a sterile cork borer, and 50 µL of each EtOH, MeOH, or A extract was added to the respective wells.

Meropenem (10 µg/well) and amphotericin B (100 µg/well) were used as positive controls for the antibacterial and antifungal assays, respectively. EtOH, MeOH, and distilled water were used as solvent controls. Bacterial plates were incubated at 37 °C for 18–24 h, whereas yeast plates were incubated at 35 °C for 24–48 h. Following incubation, the inhibition zones were measured in millimeters (mm). All experiments were performed in triplicate [37, 38, 39].

4.5.3. Determination of Minimal Inhibitory Concentration (MIC) for Bacteria

The MIC values of the plant extracts against bacterial strains were determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Bacterial inocula were prepared from fresh cultures, adjusted to the 0.5 McFarland turbidity standard, and diluted to obtain a final inoculum concentration of 5×10^5 CFU/mL. Briefly, 100 µL of cation-adjusted Mueller–Hinton broth (CAMHB) was added to each well of a sterile U-bottom 96-well microtiter plate, followed by 100 µL of the respective plant extract in the first well. Two-fold serial dilutions were then prepared. Subsequently, 5 µL of the bacterial suspension was added to each well, and the plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest extract concentration showing no visible bacterial growth. CAMHB, EtOH, MeOH, distilled water, and meropenem were included as growth, solvent, and positive controls, respectively [40]. Growth controls were performed for all bacterial strains, and CAMHB was included as a medium control. The solvents EtOH, MeOH, and distilled water were also tested under the same experimental conditions and used as negative controls. Meropenem was used as the positive control. All experiments were performed in triplicate.

4.5.4. Determination Minimal Inhibitory Concentration (MIC) for Yeast

The MIC values of the plant extracts against *C. albicans* ATCC 90028 were determined according to CLSI guidelines. *C. albicans* was cultured on SDA at 37 °C for 48 h. Subsequently, 100 µL of RPMI-1640 medium was dispensed into each well of a sterile U-bottom 96-well microtiter plate, and the plant extracts were serially diluted in RPMI-1640 medium. A yeast suspension was prepared from a fresh SDA culture, adjusted to the 0.5 McFarland turbidity standard (1×10^6–5×10^6 cells/mL), and further diluted in RPMI-1640 medium to obtain a final inoculum concentration of 5×10^2–2.5×10^3 cells/mL. Then, 100 µL of the yeast suspension was added to each well containing the serially diluted extracts. The microplates were incubated at 37 °C for 24–48 h and evaluated after 24 and 48 h. The MIC was defined as the lowest extract concentration showing no visible yeast growth. A growth control for *C. albicans* and an RPMI-1640 medium control were included. The solvents EtOH, MeOH, and distilled water were also tested under the same experimental conditions and used as negative controls. Amphotericin B was used as the positive control. All experiments were performed in triplicate [41].

4.5.5. Determination of the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

MBC and MFC values were determined from the MIC assay wells showing no visible microbial growth. A 5 µL aliquot from each selected well was inoculated onto MHA plates for bacteria and SDA plates for yeast. The bacterial plates were incubated at 37 °C for 24 h, whereas the yeast plates were incubated at 37 °C for 48 h. The MBC and MFC were defined as the lowest extract concentrations showing no microbial growth on the corresponding agar plates [42].

4.6. Statistical analysis

Statistical analyses were performed using two-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$. For each combination of sample type and extraction solvent, three independent extraction replicates were used for statistical analysis (n = 3). Each independent extract was analyzed in triplicate, and the mean of the analytical measurements was used for statistical analysis.

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