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IMPROVED EXTRACTION OF BIOACTIVE COMPOUNDS FROM THE POLLENS OF *TYPHA DOMINGENSIS* WITH SEQUENTIAL CONVENTIONAL AND ULTRASOUND TREATMENT

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ABSTRACT

Typha domingensis pollen is obtained from staminate flowers of plant that is edible. In this study, bioactive compounds from pollen were extracted using ethanol and methanol with conventional method. Further ultrasonication was applied to the samples to improve extraction efficiency. The extraction yield was higher with ethanol (26.3±0.14%) than with methanol (25.4±0.3%) and applying ultrasonication increased the yields significantly (P <0.05). Total phenolic compounds in ethanol and methanol extracts were determined as 9.83±0.48 and 9.71±0.55 mg GAE/g dry matter with the conventional method, and 11.76±0.64 and 12.74±0.37 mg GAE/g dry matter after ultrasonication, respectively. The flavonoid content with ethanol extraction was significantly higher than with methanol in both conventional and ultrasonication methods (P <0.05). Antioxidant activities using DPPH, ABTS, CUPRAC, and FRAP tests, were determined, and enhanced antioxidant capacities were observed after ultrasonication. The bioactive compounds were qualitatively analyzed using UV-VIS spectroscopy and FTIR which confirmed the presence of polyphenols.
Keywords: *Typha domingensis* pollen, extraction, ultrasonication, bioactive compounds

TYPHA DOMINGENSIS POLENLERİNDEN BİYOAKTİF BİLEŞİKLERİN EKSTRAKSİYONUNDA GELENEKSEL VE ULTRASONİK YÖNTEMLERİN ARDIŞIK KULLANIMI

ÖZ

Typha domingensis bitkisinin, erkek çiçeklerinden elde edilen polen, yenilebilir özelliktedir. Bu çalışmada, polenden biyoaktif bileşikler, etanol ve metanol kullanılarak geleneksel yöntemle ekstrakte edilmiştir. Ardından, ekstraksiyon verimliliğini artırmak için örneklere ultrasonikasyon uygulanmıştır.

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Etanol ile ekstraksiyon verimi (%26.3±0.14), metanole (%25.4±0.3) göre daha yüksek bulunmuş ve ultrasonikasyon uygulaması verimi önemli ölçüde artırmıştır (P <0.05). Etanol ve metanol ekstraklarında, toplam fenolik bileşik içerikleri, geleneksel yöntemle sırasıyla 9.83±0.48 ve 9.71±0.55 mg GAE/g, ultrasonikasyon uygulamasından sonra ise 11.76±0.64 ve 12.74±0.37 mg GAE/g kuru madde olarak belirlenmiştir. Etanol ekstraksiyonundaki flavonoid içeriği hem geleneksel hem de ultrasonikasyon yöntemlerinde metanolden önemli ölçüde yüksek olmuştur (P <0.05). Örneklerin antioksidan aktiviteleri DPPH, FRAP, ABTS ve CUPRAC yöntemleriyle belirlenmiş ve sonuçlar ultrasonikasyonun antioksidan kapasitelerini de arttırdığını göstermiştir. Biyoaktif bileşikler, UV-VIS spektroskopisi ve FTIR ile kalitatif olarak analiz edilmiş ve polifenollerin varlığı doğrulanmıştır.
Anahtar kelimeler: *Typha domingensis* pollen, ekstraksiyon, ultrasonikasyon, biyoaktif bileşikler

INTRODUCTION

Typha species, also called as cattail, are perennial, herbaceous, rhizomatous plants belonging to the Typhaceae family. Genus *Typha* is the only member of the family and consists of about 15 members (Rao and Divya, 2016; Al-Bader, 2018; Sorourian et al., 2020). *Typha* species are excessively invading and grow wildly, especially in tropical wetlands such as marshes, lagoons, and lakes, and the length can reach 2-3 m (Sorourian et al., 2020; Pandey et al., 2022). It has been also reported that the species may grow worldwide, except Antharctis, because of their high growth rates under suitable conditions such as light, water, and nutrients. In such cases, they may colonize and dominate large areas (Bansal et al., 2019; Cruz et al., 2019). *Typha domingensis* is monoecious and develops inflorescence as spikes. The inflorescence contains both staminate (male) and pistillate (female) flowers. Pistillate flowers are brown and densely packed that possess a cylindrical shape. Staminate flowers are yellow and located at the top of the plant (Bansal et al., 2019; Carvalho and Mariath, 2019). Staminate flowers can produce 216 to 4000 kg of pollens per hectare (Arenas and Scarpa, 2003).

As they are widely distributed at wetlands, people living around that area have been utilizing the different parts of the plant for various purposes since ancient times (Corneanu et al., 2014). Owing to high cellulose content (about 67%), they are used for craftwork, in the production of fabrics, making vessels, cattle feeding, the manufacture of boats in some countries (Garcia et al., 2019; Barbero-Barrera et al., 2021; John et al., 2022; Pandey et al., 2022). They are also used for medicinal treatments such as diarrhea, wounds, dysmenorrhea, nose bleeding, and burns (Akkol et al., 2011; Corneanu et al., 2014; Karbon and Alhammer, 2020; Sorourian et al., 2020). Recently, *Typha* species have been attracted for the potential utilization as biomass in the production of bio-based materials such as furfural and ethanol, and for bioremediation (Garcia et al., 2019; Eid et al., 2020; Mukthar et al., 2020; Pandey et al., 2022). Different parts of the plants such as rhizome, shoots, and pollens are edible and have been consumed as food in some countries including Southern Iraq, India, South America, China and New Zealand (Zhang et al., 2020). It is consumed either fresh or after cooking (Al-Bader, 2018). Pollen from staminate flowers of *T. domingensis* is yellow in color and used in powder form. It has high nutritional value that contains proteins (0.112-0.184 g/g), carbohydrates (0.101-0.174 g/g), vitamin C (1.176-2.319 mg/g) and minerals such as calcium, phosphorus, magnesium, and iron (Arenas and Scarpa, 2003). It also contains bioactive compounds such as phenolics and flavonoids. Characteristic golden yellow color of the pollen is in consequence of the flavonoids (Aljazy et al., 2021). Pollen may be mixed with sugar or honey and served as dessert or may be included in bread and cake formulations (Zhang et al., 2020). In southern Iraq, pollen is used to produce a sweet called as Khirret (Alzajy et al., 2021). It has been consumed by Native Americans in bread making, for thickening soup, to give yellow color to rice dishes, and a refreshing beverage is also prepared from the pollen (Linskens and Jorde, 1997; Arenas and Scarpa, 2003; Corneau et al., 2014).

Polyphenols are secondary metabolites produced by plants as a part of defense mechanism for protection from environmental damage. They include flavonoids, isoflavonoids, and non-

flavonoids which have beneficial effects on human health owing to their antioxidant properties (Hasan et al., 2017). Thus, recent studies have focused on the utilization of polyphenols in the production of functional foods and neutraceuticals after the extraction and separation from the plants (Altemimi et al., 2015). Besides conventional methods, new strategies have been introduced to improve the extraction efficiency of polyphenols from the plants, such as microwave-assisted extraction, ultrasound-assisted extraction, and supercritical fluid extraction (Altemimi et al., 2016). Ultrasound-assisted extraction is one the most attractive methods which have been widely applied in recent years because it is a fast, simple, and efficient method (Altemimi et al., 2016; Lafarga et al., 2019; Tarone et al., 2021). Applying ultrasound to the extraction medium creates cavitation where air bubbles are formed and subsequently collapse resulting in the disruption of the cells (Zafra-Rojas et al., 2020). Polyphenols are generally bounded to carbohydrates and proteins (Goula et al., 2016). Ultrasonication helps the liberation of bioactive compounds from plant matrices by breaking down these molecules (Lohani and Muthukumarappan, 2021).

In the literature, there are several studies about the extraction of phytochemicals from *T. domingensis* pollen using various methods. However, to the best of our knowledge, there is no report about the ultrasonication-assisted or sequential extraction. Therefore, in the current study, it was aimed to combine ultrasonic extraction with conventional extraction in order to improve the extraction of phytochemicals from *T. domingensis* pollen. In this context, phytochemicals were extracted using both conventional and sequential conventional and ultrasonication-assisted methods. Two extraction solvents (ethanol and methanol) were used. Total phenolic and flavonoid contents as well as antioxidant properties samples obtained using different methods were determined comparatively. Additionally, phytochemicals were also examined qualitatively with FTIR and UV-VIS spectroscopy.

MATERIALS AND METHODS

Materials

Ethanol, methanol, hydrochloric acid, potasssium persulfate, Folin-Ciocalteu phenol reagent, sodium carbonate, iron(III) chloride hexahydrate, sodium acetate, gallic acid were provided from Merck (Darmstadt, Germany). 2,2-diphenyl- 1-picrylhydrazyl (DPPH), Trolox [(±)-6- hydroxy-106 2,5,7,8-tetramethylchroman-2-carboxylic acid], Neocuproine, copper (II) chloride, ammonium acetate, 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), 2,20-azino-bis/3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and potassium persulfate were supplied from Sigma (St. Louis, MO). *T. domingensis* pollens were purchased from local market of Basrah (Iraq).

Ultrasonic-assisted and conventional extractions of the bioactive compounds from *T. domingensis*

Conventional and sequential conventional and ultrasonic-assisted extraction methods were applied in order to extract the bioactive compounds from *T. domingensis* according to the method described by Jerman et al. (2010) with some modifications. Ethanol (70%) and methanol (70%) were selected as solvents. In the conventional method, solvents (25 mL) were added to weighed samples (2.5 g), and homogenized for 5 min with ultra-turrax (IKA, T18, Germany) followed by the centrifugation at 6100 x *g* for 20 min then the supernatants were filtered. Sequential conventional and ultrasonication extraction was carried out using the same solvents and the sample amounts. Firstly, the samples were homogenized for 5 min with ultra-turrax, subsequently they were subjected to ultrasonication using a 200 W and 24 kHz ultrasonic device (Bandelin HD2200, Germany). The mixture was placed in a beaker and the probe (TS104) was immersed 1.5 cm from the surface of the solution. To keep the temperature constant (20°C±2), each beaker was held in an ice bath and the temperature was monitored. The ultrasound power and time were adjusted to 120 W and 30 min, respectively. After ultrasonication, the samples were centrifuged (6100 x g for 20 min) and the supernatants were

filtered. All the extracts were kept at 4°C until analysis.

Extraction yield

To calculate extraction yield, extracts were dried at 50°C until the weight was constant. The extraction yield was then calculated as follows:

$$Extraction\ yield\ (\%) = \frac{extraction\ weight\ (g)}{powder\ weight\ (g)} x100$$

Total phenolic content analysis

The total phenolic content of the extracts was determined according to the method described previously by Wojdylo et al. (2007). Briefly, 25 µL of extract and 75 µL of solvent were mixed with 0.2 mL of Folin–Ciocalteu reagent. One milliliter of sodium carbonate solution was added to the mixture after 3 min. Samples were incubated for 1 h at room temperature. The absorbance was recorded at 765 nm with a spectrophotometer (Shimadzu UV-1240, USA). The results were calculated as mg gallic acid equivalent (GAE) /g dry matter using a gallic acid standard curve (R^2 = 0.9986).

Total flavonoid analysis

The total flavonoid content of the extracted samples was determined using the aluminum chloride colorimetric assay. For this 100 µL sample, 300 µL distilled water and 30 µL $NaNO_2$ (5%, w/v) were mixed in a tube, and reacted for 5 min. Subsequently, 30 µL $AlCl_3$ (10%, w/v) was added followed by the addition of 200 µL NaOH (1 mM) after 5 min. Then the final volume was adjusted to 1 mL with distilled water. Absorbance was measured at 510 nm using a UV-VIS spectrophotometer. The amount of flavonoids was calculated using the standard curve which was constructed using quercetin. The results were estimated as mg quercetin equivalent (QE)/g dry matter (R^2 = 0.9997) (Egdhami and Sadeghi, 2010; Kamtekar et al., 2014).

Determination of antioxidant capacities

Antioxidant capacities of extracts were determined with four different assays, which were DPPH scavenging activity, ferric reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC) and $ABTS^{\bullet+}$ assay (Huang et al., 2005). DPPH scavenging activity assay was performed using the method of Brand-Williams et al. (1995) with some modifications. In a test tube, 50 µL of extract, 50 µL of solvent and 3 mL of DPPH solution (0.051 mmol/L) were added and mixtures were incubated for 30 min at room temperature. Trolox solutions with different concentrations were used to construct a standard curve (R^2 = 0.9986). Absorbance values were recorded at 517 nm and the results were expressed as Trolox equivalent in mg/g in dry matter.

The FRAP assay was applied according to the procedure of Benzie and Strain (1996). FRAP reagent was prepared by mixing 10 mmol/L TPTZ, 20 mmol/L $FeCl_3.6H_2O$ and 300 mmol/L acetate buffer (pH 3.6) in a ratio of 1:1:10 (v/v), respectively. The extract (50 µL) and solvent (50 µL) were transferred into a test tube. Then, 1.8 mL of FRAP reagent and 1.2 mL of distilled water were mixed and incubated at 37°C for 15 min. The results were calculated with respect to the standard curve of Trolox (R^2 = 0.9991) and expressed as mg/g in dry matter.

The ABTS assay was performed according to the method described by Re et al. (1999) $ABTS^{\bullet+}$ stock solution was prepared 16 h before use by reacting 2.45 mmol/L aqueous solution of $K_2O_8S_2$ with 7 mmol/L aqueous solution of $ABTS^{\bullet+}$. Water/ethanol mixture (1:1, v/v) was added to the stock solution until the absorbance value of the working solution was 0.70. Then, 50 µL of the extract was mixed with 3 mL of $ABTS^{\bullet+}$ working solution and incubated for 6 min at room temperature. The absorbance values were recorded at 734 nm and the results were expressed as milligram Trolox equivalent per gram dry matter (R^2 = 0.9994).

For the CUPRAC assay, 50 µL of extract and 1.05 mL of distilled water were mixed and 1.0 mL each of copper(II) chloride (10^{-2} mol/L in distilled water), neocuproine (7.5×10^{-2} mol/L, in 96% ethanol), and ammonium acetate buffer solution (in distilled water, pH 7.0) were added to complete the final volume to 4.1 mL. After

mixing, the samples were incubated at room temperature for an hour (Apak et al., 2004). Absorbance values were recorded at 450 nm and the results were expressed as Trolox equivalent in mg/g in dry matter ($R^2 = 0.9971$).

Qualitative analysis of the extracts

The extracts were monitored qualitatively by determining the spectra of supernatants between 200 and 700 nm using a UV–VIS spectrophotometer. Fourier transform infrared (FTIR) spectroscopy analyses of the dried extracts were performed on a Perkin Elmer spectrometer (Massachusetts, USA) using Spectrum Two Model by ATR technique. Data was analyzed with Spectrum Software. The spectra were obtained at room temperature in the range of 4000 and 400 cm^{-1}.

Statistical analysis

The results were statistically analyzed by ANOVA using SPSS (version 11.5, SPSS Inc., USA). To determine the differences between extracts, Duncan's multiple range test with a significance level of 0.05 was used.

RESULTS AND DISCUSSION

Extraction yields

The extraction yield basically depends on the type of extraction and the plant material. Thus, the extracts of the pollens were prepared by using two different solvents (methanol and ethanol) and two extraction methods. The extraction yields of all the conditions are depicted in Table 1. The extraction yield with ethanol (26.34±0.14%) was significantly higher than with methanol (25.38±0.31%) (P <0.05). On the other hand, further application of ultrasonication to the samples led to a significant increase in the yields for both samples (P <0.05). The increase in the extraction yields after ultrasonication using ethanol and methanol were detected as 9.5 and 12.8% respectively. The increase in yield after ultrasonication was attributed to the cavitation bubbles which disrupt the cell wall and also increases the mass transfer rate of the cell components into the extraction solvent (Altemimi et al., 2016; Goula et al., 2016). Furthermore, it aids in removing the pholyphenols from proteins and carbohydrates (Lohani and Muthukumarappan, 2021). Alhajali and Ali-Nizam (2021) reported the extraction yields of *Pistacia atlantica* as 30.12% and 24.20%, respectively. Gonelimali et al. (2018) investigated the extraction yields of various plants using water and ethanol as solvents and applied both conventional and ultrasonication-assisted methods. They reported increased extraction yield with ultrasonication.

Table 1. The extraction yields of the extracts

Extracts		Extraction yields (%)
Ethanolic	E	26.34±0.10^{b}
	EUS	29.09±0.31^{a}
Methanolic	M	25.38±0.22^{c}
	MUS	29.09±0.03^{a}

Values followed by the different letter shows significant difference within extracts. E and M are the ethanol extracts with conventional method. EUS and MUS are the ethanol and methanol extracts after ultrasonication.

Total phenolic and flavonoid contents

Total phenolic contents of the ethanol and methanol extracts from the conventional extraction were almost similar and found as 9.83±0.48 and 9.71±0.55 mg GAE/g dry matter, respectively (P >0.05). A significant increase in the total phenolic content has been detected when the samples were treated with ultrasonication (P <0.05), and they were determined to be 11.76±0.64 and 12.74±0.37 mg GAE/g dry matter, respectively (Figure 1a). Unlike the total phenolic contents, flavonoid contents were affected by the type of the solvent. Using ethanol as the extraction solvent yielded in higher flavonoids than methanol (Figure 1b). The total flavonoid amount obtained with methanol was almost half of the flavonoids from the ethanol extraction. Regarding the ultrasonication effect to the extraction of flavonoids, remarkable increases in both extraction solvents have been observed (P <0.05). The total flavonoid contents of ultrasonicated ethanol and methanol extracts were 3.40±0.17 and 2.07±0.06 mg QE/g dry matter. As mentioned before ultrasonication releases the bioactive compounds from the plant matrices which are bounded with carbohydrates

and proteins (Goula et al., 2016; Lohani and Muthukumarappan, 2021). Improved extraction of bioactive compounds from different plant materials using ultrasonication were reported by various researchers (Jerman et al., 2010; Altemimi et al., 2015; Corbin et al., 2015; Tarone et al., 2021). Altemimi et al. (2016) compared the conventional and ultrasonication-assisted extraction of phenolic compounds from peaches and pumpkins and found higher phenolic compounds and antioxidant activity with ultrasonication. They also observed the extracted samples under scanning electron microscopy and detected highly damaged cell walls with ultrasonication (Altemimi et al., 2016).

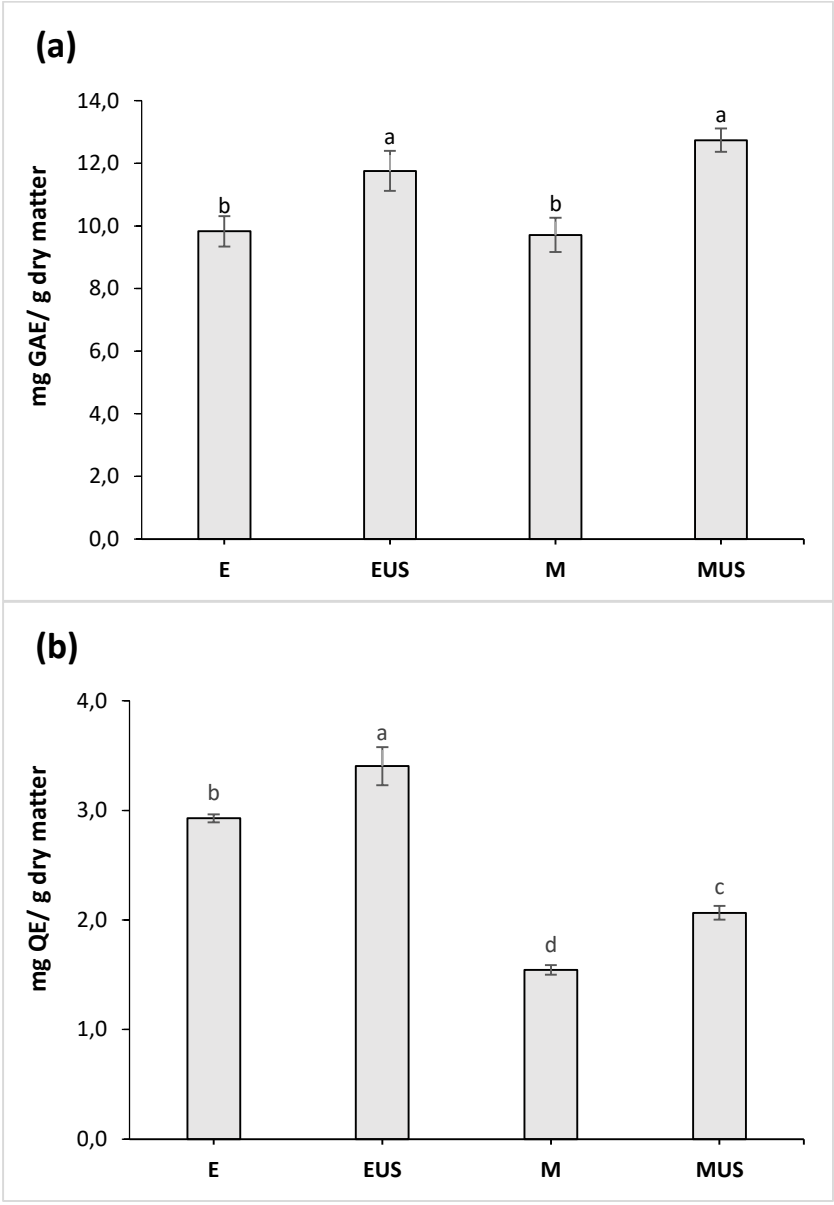


Figure 1. Total phenolic (a) and total flavonoid (b) contents of the extracts. Values followed by the different letter shows significant difference within extracts (E and M are the ethanol extracts with conventional method. EUS and MUS are the ethanol and methanol extracts after ultrasonication)

Total phenolic compounds and flavonoid content of *T. domingensis* pollen were reported by several researchers using different extraction techniques. Chai et al. (2014) determined the total phenolic and flavonoid contents of the staminate flower of *T. domingensis* in the aqueous extracts as 9.92±0.14 mg GAE and 8.16±0.12 mg QE/g dry matter, respectively. Karbon et al. (2019) determined the flavonoid content of the pollen as 1.43 mg rutin equivalent/g dry matter. Sardar et al. (2014) used various extraction solvents for the extraction of bioactive compounds from the pollen and reported the variation in the extracted polyphenols and flavonoid contents depending on the solvent type. They reported methanol as a better solvent for the extraction of polyphenol, while n-hexane was the most effective for flavonoid extraction.

Antioxidant activities

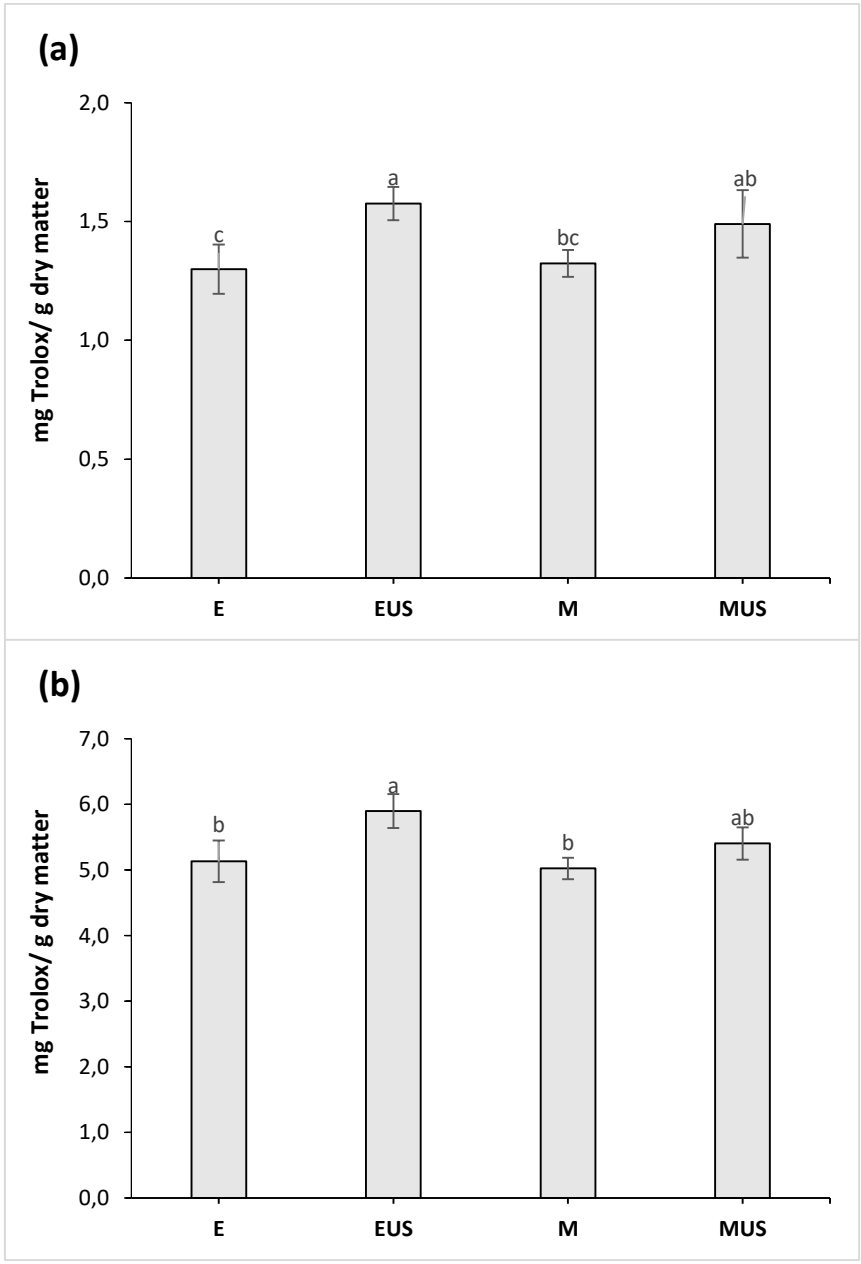
To comprehensively examine different aspects of antioxidant activities of the extracts, four different methods were applied, namely DPPH, FRAP, ABTS and CUPRAC. All of these analyses are spectrophotometric electron transfer based assays and measure the ability of an antioxidant in the reduction of an oxidant. However, they differ from each other according to their working pH, applicability to hydrophilic and lyophilic antioxidants and chromogenic redox reagents with different standard potentials. Therefore, the antioxidant potential of the extracts was evaluated with different tests (Apak et al., 2007). According to the results, DPPH values of all extracts ranged from 1.30±0.10 to 1.58±0.07 mg Trolox/g dry matter (Figure 2a). There were significant differences between the DPPH values of the extracts and the highest one belonged to ultrasonicated ethanol extract (P <0.05). Aljazy et al. (2021) examined the antioxidant activity of three pollen extracts (aqueous, ethanol, and hexane) and found that the pollen extracts showed a good ability to inhibit DPPH radical. They reported that the percentage inhibition of the extracts was not significantly different at a concentration of 75 mg/mL. Similarly, this study showed no significant differences between ethanol and methanol extraction.

When the FRAP results of the extracts were compared, the lowest value was detected as 5.02±0.16 mg Trolox/g dry matter in methanol, while the highest value was 5.90±0.26 mg Trolox/g dry matter in ultrasonicated ethanol extract (Figure 2b). The FRAP values were not affected by the type of solvents. In the study of Sardar et al. (2014), the effect of different solvents on the antioxidant potential of the pollen of *T. domigensis* was detected with FRAP assay. It was reported that solvent type affected the FRAP assay and the highest value was obtained in methanol extract, which is in contrast with the results of this study.

The ABTS values of the ethanol and methanol extracts from the conventional extraction were almost similar and determined as 3.67±0.09 and 3.66±0.03 mg Trolox/g dry matter, respectively (Figure 2c). The ABTS activity significantly increased after ultrasonic treatment (P <0.05) and the values were detected as 4.06±0.08 mg Trolox/g dry matter for ethanol extract and 3.90±0.07 mg Trolox/g dry matter for methanol extract. Dilshad et al. (2022) conducted a study evaluating phytochemical potential of methanol extract and n-hexane fraction of *T. domingensis*. They found the ABTS value of methanolic extract as 114.31 mg Trolox/g dry matter, which is higher than this study. One important reason could be the differences in extraction times which was longer than our study. The relative antioxidant activities determined by DPPH scavenging, FRAP and ABTS were in agreement (Figure 2c).

CUPRAC is another antioxidant activity method and the principle is that the Cu (II)-neocuprine complex converts the Cu (I)-neocuprine form at the end of the reaction. In this study, CUPRAC values of the ethanol and methanol extracts were 11.80±0.14 and 9.76±0.55 mg Trolox/g dry matter, respectively, which showed the significant effect of the solvent type (Figure 2d) (P <0.05). Moreover, ultrasonicated ethanol extract had the highest CUPRAC value (12.01±0.40 mg Trolox/g dry matter). When the results of the antioxidant activities were generally examined, much higher values were detected in the samples treated with ultrasonication than in conventional groups. The

phenolic and flavonoid contents of *T. domingenses* extract provide high antioxidant activity by scavenging free radicals with the help of hydrogen atoms of these compounds (Aryal et al., 2019). Since these bioactive compounds were released more from plant matrices during ultrasonication, significant increases in antioxidant activities were detected after treatment.



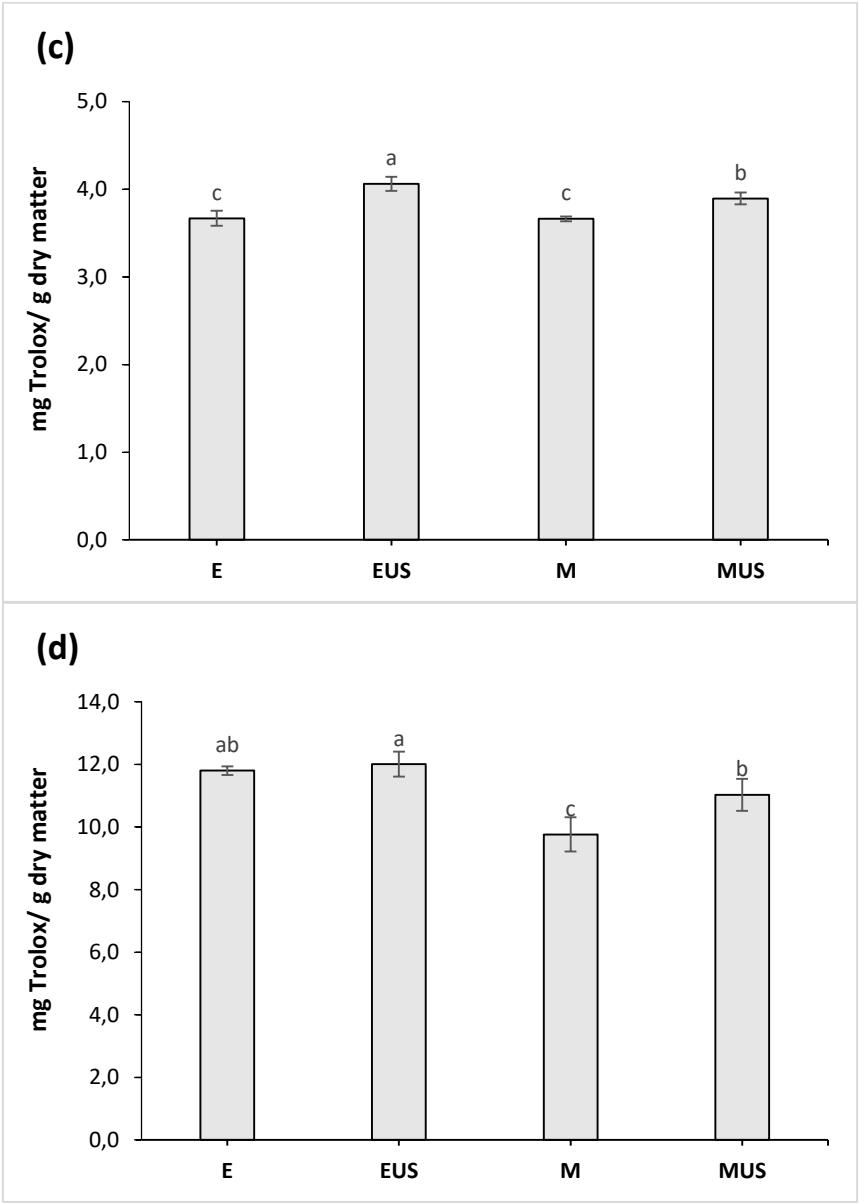


Figure 2. DPPH (a), FRAP (b), ABTS (c) and CUPRAC (d) contents of the extracts. Values followed by the different letter shows significant difference within extracts (E and M are the ethanol extracts with conventional method. EUS and MUS are the ethanol and methanol extracts after ultrasonication).

Qualitative determination of phenolic and flavonoids with UV-VIS spectroscopy and FTIR

UV-VIS absorption spectra of the extracts were monitored at 200-700 nm to determine specific absorption wavelengths (Figure 3). There was no absorption at the wavelengths over 400 nm. Main peaks were detected at around 341-347, 261-266, and 200-225 nm. All the samples had similar peaks, however, their intensities differed which were attributed to the different amounts of the substances. It is known that the phenolic and flavonoids absorb the UV light between 200 and 350 nm which is exerted due to the n–π* electronic transition of aromatic substances as well as chromophores (Rafi et al., 2018; Song et al., 2020).

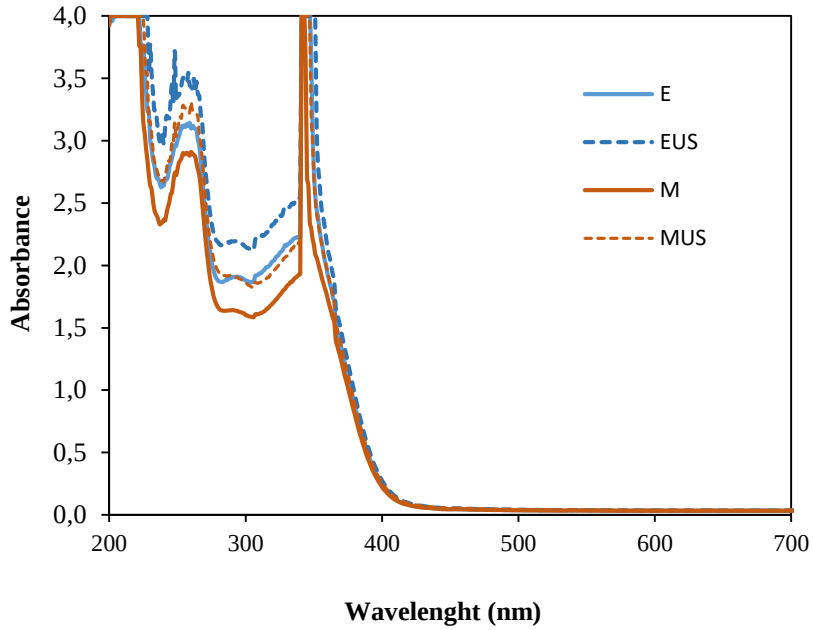


Figure 3. UV-VIS absorption spectra of the ethanol and methanol extracts of *T. domingensis* pollen. E and M are the ethanol extracts with conventional method. EUS and MUS are the ethanol and methanol extracts after ultrasonication.

Fourier transform infrared spectroscopy is a useful tool for the identification of main functional groups and chemical bonds in biological molecules, hence it may provide insight into the presence of certain compounds (Palacio et al., 2014). In the current study, the extracts were dried and subjected to FTIR analysis to confirm the presence of phenolic compounds and flavonoids by detecting the functional groups associated with those molecules (Figure 4a and b). The identical peaks were obtained with the same solvents before and after ultrasonication. However, the intensities of the peaks increased after ultrasonication in both ethanol and methanol extracts that were related with the amount of the chemical compounds in the samples. As the transmittance value is low, absorbance is high which means that the sample contains a higher amount of compounds. The transmittance intensities obtained with FTIR were coherent with the results of the phytochemical analysis performed before and after ultrasonication. The wide peaks between 3600 and 2990 cm^{-1} were detected in all the samples which were attributed to the stretching vibration of –OH groups in phenols and flavonoids (Kalaichelvi and Dhivya, 2017). The peaks at 2926 and 2852 cm^{-1} were associated with the –CH stretching vibrations of aromatic rings (Jain et al., 2016; Collazos-Escobar et al., 2020). The peak at 2852 cm^{-1} was more apparent in the ethanol extracts than in methanol extracts. The peaks between 1734, and 1600 cm^{-1} were due to the vibrations and stretching of –C=O groups in flavonoids (Kumar and Roy, 2018). The highly intense peaks at 1043 cm^{-1} were associated with C-O-C stretch (Jain et al., 2016). The peaks between 987 and 543 cm^{-1} were attributed to the angular deformation of C-H bonds in aromatic rings (Batista et al., 2016). The presence of -OH, -C=C-, and C-O-C groups confirmed that the extracts contained phenolics and flavonoids (Kumar and Roy, 2018). Thus, existence functional groups and chemical bonds related with polyphenols and flavonoids validated the presence of those molecules.

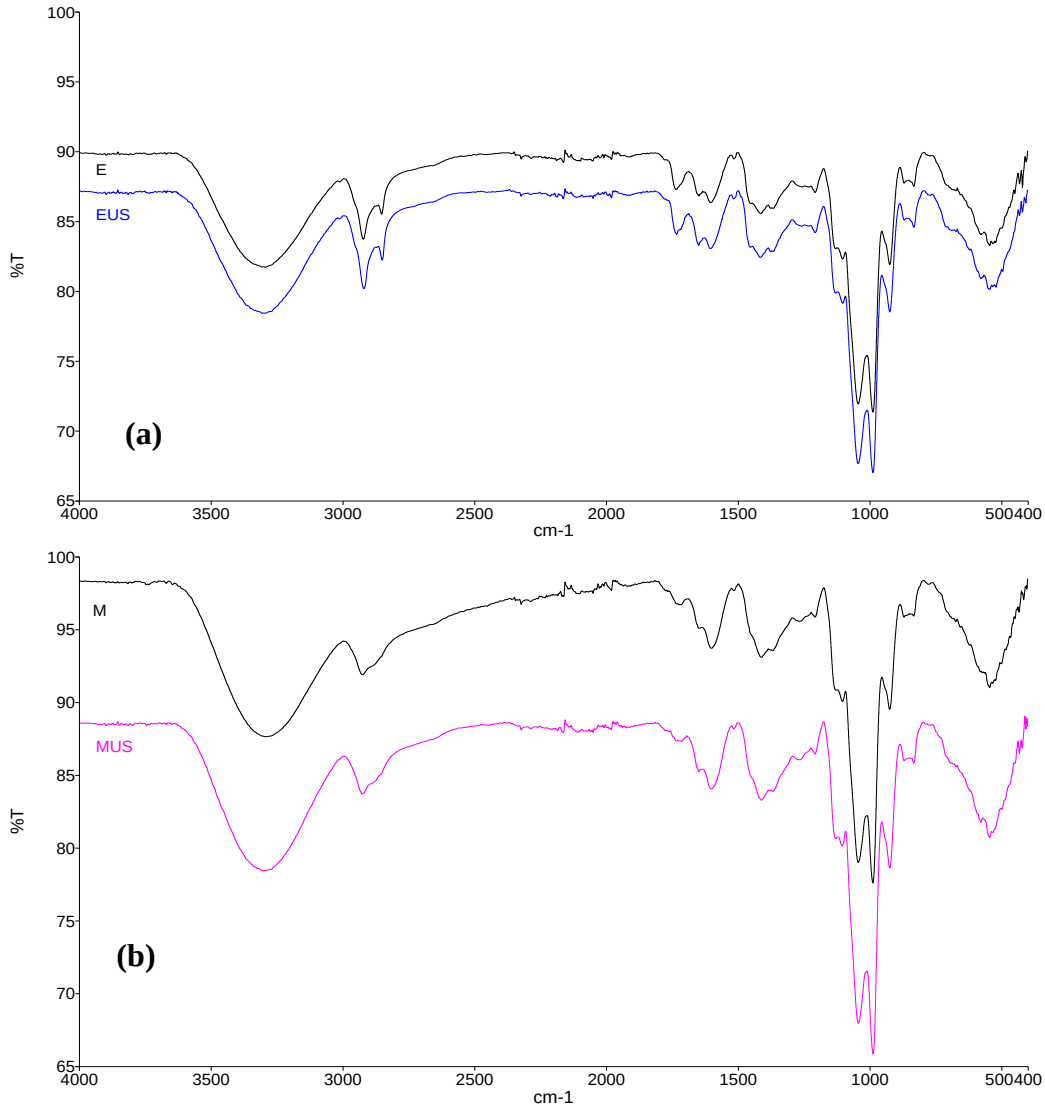


Figure 4. FTIR spectra of ethanol (a) and methanol (b) extracts of *T. domingensis* pollen. E and M are the ethanol extracts with conventional method. EUS and MUS are the ethanol and methanol extracts after ultrasonication.

CONCLUSIONS

T. domingensis pollen which is included in the traditional dishes of some culture were tested for its bioactive compounds. Conventional extraction method was compared with the sequential conventional and ultrasonicated extraction using ethanol and methanol as extraction solvents. Sequential conventional and ultrasonicated extraction significantly improved the extraction yield and the amount of bioactive compounds. In spite of similar total phenolic contents with ethanol and methanol, the total flavonoids were substantially higher in methanol extracts than methanol extracts. In addition, sequential extraction resulted in higher antioxidants activities in all conditions which were determined using four different methods (DPPH, ABTS, FRAP and CUPRAC assays). Ethanol extracts exhibited higher antioxidant activities than methanol extracts that indicated the ethanol was better than methanol for the extraction of bioactive compounds from *T. domingensis* pollen. FTIR analyses confirmed the presence of phenolic compounds and flavonoids. It also

indicated that ultrasonication could only increase the amount bioactive compounds, but did not provide the extraction of different compounds.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHORS' CONTRIBUTIONS

Ayşe Avcı: Conceptualization, supervision, methodology, investigation, formal analysis, writing-review & editing. İnci Cerit: Conceptualization, methodology, investigation, formal analysis, writing-review & editing. Mohammed Hamk: Conceptualization, investigation, formal analysis. Semra Yılmazer Keskin: Formal analysis, review & editing. All authors read and approved the final manuscript.

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