

Research Article

Comparison of Varied-Frequency Ultrasound and Conventional Extraction on Antioxidant Properties and Phytochemicals From *Cinnamomum verum* Bark

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Cinnamomum verum is a well-renowned medicinal plant among the scientific community owing to its unique flavoring profile and presence of bioactive constituents. Nonetheless, an optimized ultrasound-assisted extraction (UAE) method with special reference to varied-frequency ultrasound for effective extraction of phenolic compounds from *C. verum* bark still lacks. Therefore, present research was conducted to evaluate the impact of varied frequency (25, 40, and 59 kHz) US on the content of total phenolics, flavonoids, antioxidant potentials [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC)], and six main bioactive compounds in *C. verum* bark. In this study, UAE at 40 kHz resulted in highest content of total phenolics (317.98 mg GAE/g), total flavonoids (49.98 mg QE/g), DPPH (82.54%), and ABTS (86.12%)-scavenging potential followed by UAE at 59 kHz, UAE at 25 kHz, and conventional extraction, respectively. A similar trend was observed for FRAP and ORAC. UAE at 49 kHz resulted in highest (FRAP: 8.12 mmol Fe²⁺/g; ORAC: 8991 μmol Trolox/g), while conventional extraction resulted in the lowest (FRAP: 5.98 mmol Fe²⁺/g; ORAC: 8116 μmol Trolox/g). The maximum content of six major bioactive compounds (Scopoletin: 7.11 mg/kg, o-coumaric acid: 23.96 mg/kg, coumarin: 4821 mg/kg, cinnamic acid: 421.98 mg/kg, cinnamaldehyde: 22198 mg/kg, and cinnamyl alcohol: 529.12 mg/kg) in *C. verum* extract was observed with UAE at 40 kHz. Conclusively, among all experimented ultrasound frequencies, US at 40 kHz showed potential to increase the content of biologically active compounds in corresponding extract. Further studies must be designed focusing on the use of greener solvents and to optimize the extraction process using multiple extraction parameters simultaneously for sustainable, safe, and efficient industrial applications.

Keywords: bioactive compounds; *Cinnamomum verum*; extraction; multifrequency ultrasound

1. Introduction

Since prehistoric times, mankind is known to rely on plants for the basic necessities of life including food, shelter, clothing, medicine, and transportation. Medicinal plants are considered as a vital component in various traditional medicine

systems such as Unani, Ayurveda, and traditional Chinese medicine for the alleviation of different ailments [1–4]. Across the globe, a recent surge has been observed in scientific community to explore medicinal plants as an alternative to synthetic medicine because of increased consumer acceptability and their potential to treat different ailments [5].

Evidently, plant extracts are source of natural antioxidants, which are being extensively employed in nutraceutical industry owing to their therapeutic potentials [6]. In recent times, the interest of scientists as well as consumers has increased in nutraceuticals owing to their therapeutic potentials and safe nature. Numerous studies support the fact that nutraceuticals possess significant therapeutic effects against different ailments [7–9].

Cinnamomum verum (true cinnamon tree) is a medicinal plant that belongs to family Lauraceae and is historically known to mankind as one of the oldest and most used spices owing to its unique flavoring and medicinal properties [10, 11]. This plant is native to Sri Lanka and South Indian regions that can grow up to 7–10 m in height [12]. Different parts of this plant mainly bark, roots, and leaves are rich source of various bioactive compounds. Main constituents present in cinnamon bark are cinnamaldehyde, eugenol, cinnamic acid, cinnamyl acetate, camphor, and cinnamyl alcohol [13]. *C. verum* comprises of diverse micro- and macronutrients including proteins, carbohydrates, minerals (calcium, zinc, manganese, magnesium, and iron), and vitamins (A, C, B3, and K) [14]. Extracts of cinnamon bark contain a cache of biologically active components such as cinnamaldehyde, cinnamic acid, vanillic acid, coumarin, and cinnamyl alcohol [5, 15, 16]. Regardless of being a spice, literature reveals that *C. verum* bark and its extracts possess wide range of health-promoting benefits. Since ancient times, the bark of this plant has been extensively used for managing several health conditions like asthma, digestive diseases, inflammation, stress, and infections [17]. Moreover, during the last few decades, several studies have been carried out to validate the potential of cinnamon's extract as antimicrobial, antidiabetic, antiinflammatory, antioxidant, cardioprotective, antitumor, and antifungal agent [5, 17–19].

Extraction is considered as a key first step to conduct chemical analysis, assess the bioactivity, and develop functional products. Extracts obtained from different parts of plants using various extraction methods contains primary and secondary metabolites. Conventionally, plant extracts were obtained using infusion, maceration, decoction, enflourage, percolation, and hydrodistillation. Even though these methods were helpful in extracting bioactive compounds from different parts of plants, these also had certain limitations. These limitations mainly include low selectivity, time consuming, the need of extensive solvent volumes, and poor extraction yield [20, 21]. In recent years, various innovative green extraction processes have been investigated by the scientists and employed by the industry to overcome the problems associated with conventional extraction. These novel nonconventional extraction techniques mainly include supercritical fluid extraction, ultrasound-assisted extraction (UAE), high pressure hot water extraction, pulsed electric field extraction, and microwave-assisted extraction. UAE has an advantage over other nonthermal extraction techniques owing to its eco-friendly nature, lesser energy requirements, reduced extraction time, and less extracting temperature [22]. During the last decade, the use of ultrasound has attained a predominant position in the scientific

community as a promising alternative to conventional extraction techniques for separation of bioactive metabolites.

Even though literature have reported methanol as one of the most suitable solvent for extraction of phenolics from plant matrices through application of ultrasound, no systematic study has been reported that compares the effect of varied frequencies (25 kHz, 40 kHz, and 59 kHz) of ultrasound on extraction efficiency from *C. verum* bark. Therefore, the current study was designed to understand the impact of varied-frequency ultrasound, which may significantly improve the extraction yield and biological activity of specific phytoconstituents. Purposely, in this study, we evaluated the effects of varied-frequency ultrasound (25, 40, and 59 kHz) extraction on total phenolic contents (TPC), total flavonoid contents (TFC), antioxidant properties (DPPH, ferric reducing antioxidant potential [FRAP], ABTS, and oxygen radical absorbance capacity [ORAC]), and six major bioactive compounds (high pressure liquid chromatography [HPLC]) in *C. verum* as compared with conventional extract.

2. Materials and Methods

2.1. Plant Material. The bark of mature *C. verum* was procured from local herbal market of Lahore, Pakistan in a single event in April 2023. We procured the bark of *C. verum* from various retailers at the same time. The procured bark was then pooled to minimize the variability. Prof. Dr. Moazam Rafiq Khan, University of Agriculture Faisalabad, Pakistan identified and validated the plant. The plant was placed in the Herbarium of the University of Agriculture Faisalabad, Pakistan against voucher specimen number UAF/125-2023 in dark/low light cabinets under controlled conditions (temperature: 20°C, relative humidity: 50%).

2.2. Chemicals. The chemicals used in analyzing TPC (gallic acid, sodium carbonate, Folin–Ciocalteu reagent), TFC (quercetin, distilled water, sodium hydroxide, aluminum chloride, and sodium nitrite), DPPH assay (methanol and 2,2-diphenyl-1-picrylhydrazyl), ABTS assay (potassium persulfate, ethanol, deionized water, and 2,2'-azobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), FRAP assay (acetate buffer, iron (III) chloride hexa-hydrate, HCl, ferric chloride, iron(II) sulfate heptahydrate, and 2,4,6-tri(2-pyridyl)-s-triazine), and ORAC assay (2,2'-Azobis(2-methylpropionamidine) dihydrochloride, fluorescein, and phosphate buffer) were purchased from Sigma-Aldrich (Germany). The standards used in HPLC analysis were procured from Sigma-Aldrich (Germany).

2.3. Preparation of *C. verum* Bark Powder. Initially, the pooled bark samples of *C. verum* were washed thoroughly with distilled water to remove the unwanted foreign particles like dirt and dust. Afterwards, the bark was shade-dried until the constant weight at 25°C–30°C for 7 days. The dried bark was ground through laboratory scale mill (Retsch GM-200) to collect resultant homogenized bark powder. The bark powder was sieved through 50-mesh sieve. The homogenized bark powder was then divided into three independent

portions, as biological replicates in this study, and was stored in polythene bags until further analysis. So, three independent replicates were performed in the following extraction processes (conventional or multifrequency ultrasound) for each treatment group.

2.4. Conventional Extraction. In order to collect the conventional extract, the *C. verum* bark powder (20 g) was extracted with methanol (500 mL) using mechanical shaker for 8 h. After extraction, the solution was centrifuged for 10 min and filtered using muslin cloth followed by vacuum filtration. The extraction solvent was removed from a resultant crude extract using rotary concentrator at 45°C. The concentrated extract at refrigeration temperature (4°C) was stored until further analysis.

2.5. Multifrequency Ultrasound Extraction. Initially, the bark powder of *C. verum* (5 g) was added to methanol (50 mL) in a 100-mL flask and was thoroughly mixed. Afterwards, this mixture was placed in a multifrequency sonication assembly (SB-600DTY, Ningbo Scientz Biotechnology Company Limited, Ningbo, China) for the UAE of *C. verum* bark powder separately at different frequencies (25, 40, and 59 kHz) with slight modification in the protocols adopted by Khursheed et al. [23]. Throughout the extraction process, a water circulation system was used to regulate and maintain the temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) of each sample. Other operating conditions of sonication were 500 W ultrasonic power and 20 min UAE time. After 20 min of sonication, each sample was separately centrifuged at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ followed by filtration of respective supernatants using 0.22 μm polytetrafluoroethylene membrane fibers. Finally, each extract was employed to rotary concentrator at 45°C and stored at refrigeration temperature (4°C) until further analysis. The extraction parameters like solvent volume, time, and temperature were chosen based on some previous studies regarding optimization of extraction efficiency from different parts of cinnamon. These studies have reported suitable extraction conditions for time and temperature ranging from 15 to 45 min and 30°C to 50°C, respectively, without any significant degradation of phenolic compounds [24–27]. Moreover, methanol was preferred as an extraction solvent because of its significant potential to solubilize polyphenols, as supported by prior published literature [28–31]. Furthermore, the solvent was used mainly for analytical purposes and not for direct application in food or food product development.

For each treatment group, homogenized bark powder was divided into three independent biological replicates and extraction along with analysis including TPC, TFC, antioxidant assay, and HPLC that were performed independently on each portion.

2.6. Analyses of TPC. TPC in both conventional extract and ultrasonically extracted *C. verum* were estimated by following the methods described by Khalil et al. [32, 33]. In this assay, 125 μL of each extract was thoroughly mixed with 500 μL of distilled water followed by the addition of Folin–Ciocalteu reagent (125 μL). A stay time of 10 min was given

to the resultant solution before the addition of 7% sodium carbonate (125 μL). The prepared mixture was again given a stay time of 1 hour and 30 min. Finally, each extract was subjected to UV-Vis spectrophotometer to evaluate the absorbance at 765 nm. Experiments were conducted in triplicates, and final results were expressed as mg GAE (gallic acid equivalent)/g.

2.7. Analyses of TFC. TFC in both conventional extract and ultrasonically extracted *C. verum* were estimated by following the methods described by Borrás-Enríquez et al. [34]. Purposely, 100 μL of each extract was separately mixed with mixture (1325 μL) that was already prepared by adding 5% sodium nitrite (75 μL) in distilled water (1250 μL) followed by incubation for 5 min. After initial incubation, 150 μL of aluminum chloride (10%) was added, and the resultant solution was again incubated for 5 min. Afterwards, another mixture (440 μL) that was already prepared by adding 1 M sodium hydroxide (500 μL) in distilled water (425 μL) was added to the incubated solution in the previous step. Finally, each extract was subjected to UV-Vis spectrophotometer to evaluate the absorbance at 510 nm. Experimentation was conducted in triplicates, and final results were expressed as mg QE (quercetin equivalent)/g.

2.8. Analyses of Antioxidant Properties

2.8.1. DPPH Scavenging Properties. The DPPH radical scavenging potential of *C. verum* extracts were assessed by performing the methods reported by Ray et al. [35]. In this assay, extracts (1000 μL) were mixed thoroughly with DPPH solution (0.1 mM) followed by incubation for 30 min at room temperature. A blank sample was also prepared containing only DPPH and methanol. Finally, the absorbance of both blank and sample containing solutions were assessed spectrophotometrically at 517 nm. Herein, the experimentation was conducted in triplicates, and final results were expressed in percentages using below formula:

Percentage of inhibition

$$= (1 - \text{sample absorbance/control absorbance}) \times 100$$

2.8.2. ABTS Scavenging Properties. ABTS radical scavenging properties of *C. verum* extracts were assessed by performing the methods reported by Dudonne et al. [36]. Deionized water was added in ABTS until the concentration reached 7 mM. ABTS solution was mixed with potassium persulfate (2.45 mM) and incubated for 12 h to generate ABTS radicals ($\text{ABTS}^{+\cdot}$). Afterwards, ABTS solution (3 mL) was mixed thoroughly with each extract (100 μL) separately and the absorbance was noticed at 734 nm. Similarly, the absorbance of a blank sample containing ethanol and $\text{ABTS}^{+\cdot}$ radical solutions was also assessed at 734 nm. Experimentation was conducted in triplicates and finally, the results were expressed in percentages using below formula:

Percentage of inhibition

$$= (1 - \text{sample absorbance/control absorbance}) \times 100$$

2.8.3. FRAP Analysis. FRAP of *C. verum* extracts were analyzed by adopting the methods reported by Dudonne et al. [36]. In this assay, The FRAP reagent was formulated by mixing acetate buffer (300 mM, pH: 3.6) in TPTZ (10 mM) prepared with HCl (40 mM) and ferric chloride (20 mM) in a ratio of 10:1:1. Different concentrations of ferrous sulfate heptahydrate were used for the preparation of standard curve. FRAP reagent (3 mL) was mixed with each extract (100 μ L) and deionized water (300 μ L) followed by incubation for 30 min. A blank sample used in this assay contained all other reagents except the extract. The absorbance of both blank and extract containing solutions were noted at 593 nm. Experimentation was conducted in triplicates and the results for FRAP were expressed as mmol Fe²⁺/g of sample.

2.8.4. ORAC Analysis. In order to determine the radical scavenging properties of *C. verum* extracts through ORAC assay, the methods reported by Dudonne et al. [36] were followed in this analysis. For this, each extract solution (300 μ L) was added to 70 nM of fluorescein in a cuvette and incubated for 5 min. Afterwards, 12 mM of APPH solution (900 μ L) was added to it and the fluorescence was noted at 485 (excitation wavelength) and 530 nm (emission wavelength), respectively, for 60 min. Blank sample in this assay comprised of phosphate buffer (300 μ L). Different concentrations of Trolox (1–7 μ M) were used for the preparation of standard curve. Experimentation was conducted in triplicates and ORAC values in this assay were estimated as μ mol TE (Trolox equivalents)/g of each *C. verum* extract.

2.9. Quantification of Major Phenolics (HPLC). Six major phenolic compounds present in *C. verum* extracts collected conventionally and through multifrequency ultrasound were analyzed using Waters 2690 HPLC system by following the protocols described by Ding et al. [37]. Purposely, the HPLC system was equipped with a UV-Vis detector (2487 Waters, 265 nm). Moreover, Waters-HPLC system comprised of a C18 column having particle size of 5 μ m and internal diameter as 4.6 $\times$ 250 mm. Details for gradient elution of mobile phase comprising of two solvents, that is, phosphoric acid (0.1%) in water as solvent A and acetonitrile as solvent B were as follows: 0 min, 90% (A) and 10% (B); 12 min, 80% (A) and 20% (B); 35 min, 50% (A) and 50% (B); 45 min, 25% (A) and 75% (B); 50 min, 0% (A) and 100% (B), and held the 100% (B) for 5 min. Standards for 6-methoxy-7-hydroxycoumarin (Scopoletin), o-coumaric acid, coumarin, cinnamic acid, cinnamaldehyde, and cinnamyl alcohol were diluted in methanol at different concentrations (0.5–1.5 mg mL⁻¹) for quantification of compounds against established standard curves. HPLC experimentation was conducted in triplicates.

2.10. Statistical Analysis. All the experimentations in this study were performed in triplicates and results were presented as mean $\pm$ S.D. For the assessment of level of significance ($p < 0.05$), one-way ANOVA was performed using Statistix software (Ver. 8.1.) after which Tukey's HSD was conducted for mean pairwise comparison.

3. Results

3.1. Impact on TPC and TFC. TPC and TFC were analyzed in conventional and varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark extracts. Figure 1a,b shows the effect of conventionally and varied-frequency ultrasound (25, 40, and 59 kHz) extracted treatments on TPC and TFC in resultant *C. verum* extracts. Results reveal that maximum total phenolics (317.98 mg GAE/g) and flavonoids (49.98 mg QE/g) were present in extracts that were ultrasonically extracted at 40 kHz. While, the least TPC (287.72 mg GAE/g) and TFC (38.14 mg QE/g) were observed in conventional extract of *C. verum*. As compared with conventional extraction, all the experimented treatments (varied-frequency ultrasound) had significant effect on TPC and TFC in *C. verum* extracts. Moreover, among all varied-frequency ultrasound treatments, as compared with US-25 kHz treatment, TPC and TFC in US at 40 kHz differ significantly ($p = 0.0135$ and $p < 0.0001$), respectively. However, TPC differed nonsignificantly ($p = 0.4656$) between US-40 kHz and US-59 kHz treatments. On the other hand, TFC were found to differ significantly ($p = 0.0003$) among US-40 kHz and 59 kHz treatments.

3.2. Impact on DPPH and ABTS Scavenging Properties. Figure 2a,b reveals the impact of conventionally and varied-frequency ultrasound (25, 40, and 59 kHz) extracted treatments on DPPH and ABTS scavenging activities in subsequent *C. verum* extracts. Ultrasonically treated *C. verum* extracts at 40 kHz showed highest DPPH and ABTS scavenging activities, that is, 82.54% and 86.12%, respectively. Whereas, minimum DPPH (70.21%) and ABTS (76.11%) scavenging activities were reported in the case of conventional extract of *C. verum*. US-40 kHz and US-59 kHz had significant (DPPH: $p = 0.054$ and $p = 0.0360$ and ABTS: $p = 0.0162$ and $p = 0.0306$) effect on DPPH and ABTS scavenging activities, respectively. Whereas, US-25 kHz had nonsignificant ($p = 0.2632$ and $p = 0.4789$) impact on DPPH and ABTS scavenging activities, respectively. Likewise, as compared with US-40 kHz, DPPH and ABTS scavenging activities of US-25 kHz and US-59 kHz also differed nonsignificantly ($p > 0.05$) (Figure 2a,b).

3.3. Impact on FRAP and ORAC Properties. Results of FRAP and ORAC properties in conventional and varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark extracts are demonstrated in Figure 3a,b. Maximum FRAP (8.12 mmol Fe²⁺/g) and ORAC (8991 μ mol Trolox/g) values were noticed in ultrasonically extracted *C. verum* bark extracts at 40 kHz whereas, minimum values (FRAP: 5.98 and ORAC: 8116 μ mol Trolox/g) were seen in conventional extract. It is evident from Figure 3a that as compared with conventional extract, varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark extracts had significant increase (US-25 kHz: $p = 0.0060$, US-40 kHz: $p < 0.001$, and US-59 kHz: $p < 0.001$) in FRAP values. On the other hand, only US-40 kHz showed significant ($p = 0.0252$) increase in ORAC values as compared with conventional treatment.

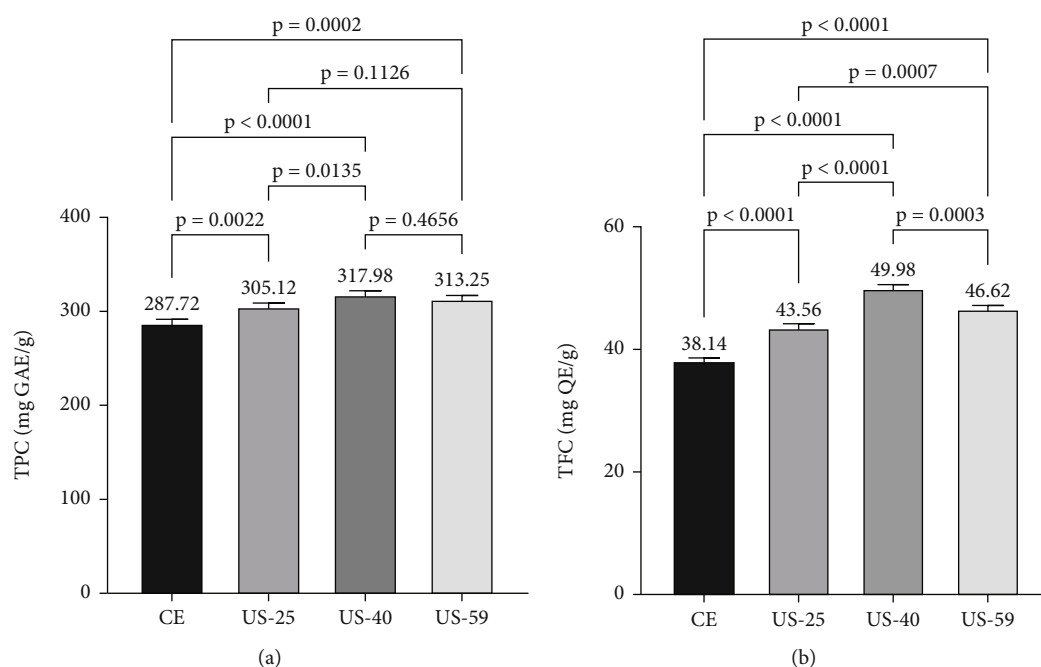


FIGURE 1: Impact of conventional and varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark extracts on total phenolic contents (TPC; (a)) and total flavonoid contents (TFC (b)). CE: conventional extraction, US-25: ultrasound-assisted extraction (25 kHz), US-40: ultrasound-assisted extraction (40 kHz), US-59: ultrasound-assisted extraction (59 kHz).

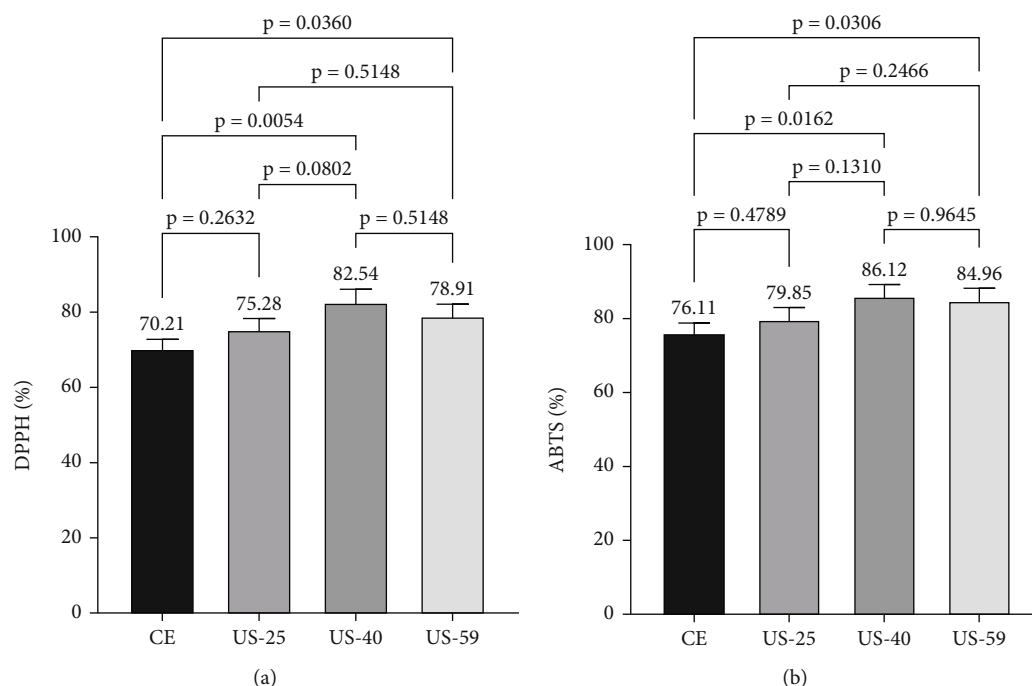


FIGURE 2: Impact of conventional and varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH (a)) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS (b)) scavenging activity. CE: conventional extraction, US-25: ultrasound-assisted extraction (25 kHz), US-40: ultrasound-assisted extraction (40 kHz), US-59: ultrasound-assisted extraction (59 kHz).

3.4. Impact on Six Major Bioactive Compounds in *C. verum* Extracts (Conventional and US). Herein, six major bioactive constituents present in conventional and varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark

extracts were compared. Table 1 shows the content of 6-methoxy-7 hydroxycoumarin (Scopoletin), o-coumaric acid, coumarin, cinnamic acid, cinnamaldehyde, and cinnamyl alcohol in conventional and ultrasonically extracted *C.*

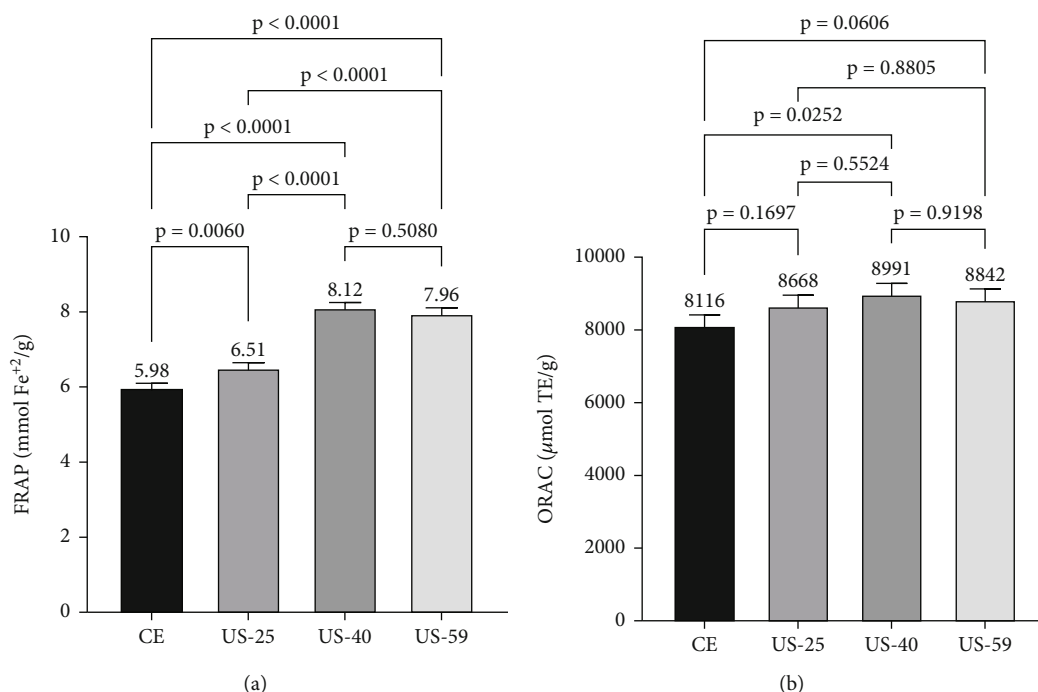


FIGURE 3: Impact of conventional and varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark extracts on ferric reducing antioxidant potential (FRAP (a)) and oxygen radical absorbance capacity (ORAC (b)). CE: conventional extraction, US-25: ultrasound-assisted extraction (25 kHz), US-40: ultrasound-assisted extraction (40 kHz), US-59: ultrasound-assisted extraction (59 kHz).

TABLE 1: Content (mg/kg) of six main bioactive compounds in conventional and varied-frequency ultrasound (25, 40, and 59 kHz) extracted *C. verum* bark extracts.

Treatments	Compounds					
	S	o-CA	C	CA	CmA	CyA
CE	4.36 ± 0.51 ^b	14.96 ± 1.12 ^c	2211 ± 145 ^c	371.16 ± 10.56 ^b	18891 ± 438 ^b	487.56 ± 9.15 ^b
US-25	6.16 ± 0.42 ^a	19.23 ± 1.06 ^b	3577 ± 156 ^b	395.65 ± 11.21 ^{ab}	19112 ± 485 ^b	512.98 ± 9.8 ^{ab}
US-40	7.11 ± 0.47 ^a	23.96 ± 1.21 ^a	4821 ± 151 ^a	421.98 ± 12.32 ^a	22198 ± 467 ^a	529.12 ± 10.21 ^a
US-59	6.78 ± 0.39 ^a	22.05 ± 1.14 ^{ab}	3965 ± 149 ^b	406.21 ± 10.98 ^a	21941 ± 431 ^a	521.85 ± 9.9 ^a

Note: Different letters in the same column show significant difference ($p < 0.05$) among the treatments.

Abbreviations: C: coumarin, CA: cinnamic acid, CE: conventional extraction, CmA: cinnamaldehyde, CyA: Cinnamyl alcohol, o-CA: o-coumaric acid, S: Scopoletin, US-25: ultrasound-assisted extraction (25 kHz), US-40: ultrasound-assisted extraction (40 kHz), US-59: ultrasound-assisted extraction (59 kHz).

verum bark extracts at varied frequencies. The content of these six compounds were expressed as mg/kg in Table 1. Among varied-frequency ultrasound (25, 40, and 59 kHz) extracts, the maximum content of all six major bioactive compounds were recorded in US at 40 kHz followed by US at 59 kHz, and 25 kHz, while the least was noticed in the case of conventional treatment.

4. Discussion

Phytochemical constituents present in plant extracts vary based on the part of plant from which they are extracted, extraction method employed, and polarity of solvent being used for extraction. Different extraction techniques have been used for extraction of bioactive constituents from *C. verum* [38–41]. Literature reveals that quality and quantity of chemical constituents present in plant may be affected

by various factors including harvesting time, maturity index, plant species, extracting conditions, and geological origin [42, 43]. Among all extraction techniques, UAE is the most commonly employed extraction technique by scientific community because of its high selectivity towards bioactive compounds, lesser extracting time, reduced energy consumption, and environmentally friendly nature [22]. UAE has been one of the most successful extraction processes used by various industries including pharmaceutical, cosmetic, and food for effective extraction of bioactive constituents from different parts of plants [44]. For enhanced extraction efficiency, optimization of extraction conditions in UAE such as ultrasound power, ultrasound temperature, ultrasound amplitude, ultrasound frequency, type, and concentration of extraction solvent are considered vital. Among all these parameters, the choice of ultrasound frequency plays the most vital role in increasing extraction efficiency, as it directly correlates with

the bubble size developed during the process [45]. Outcomes of this investigation may have a unique contribution in the field of extraction through assessing the effectiveness of ultrasound extraction at three varied US frequencies, hence, filling the current research gap concerning frequency optimization in extraction of bioactive constituents from *C. verum* bark. Identifying optimum frequency for effective UAE can be helpful in providing applied understandings and methodological advancement for nutraceutical industry and scientific community working on green novel extraction techniques. Therefore, in the present study, we evaluated the effects of varied-frequency ultrasound (25, 40, and 59 kHz) extraction on TPC, TFC, antioxidant properties (DPPH, FRAP, ABTS, and ORAC), and major bioactive compounds (HPLC) in *C. verum* as compared with conventional extract.

In the current study, we used varied-frequency (25, 40, and 59 kHz) ultrasound for extraction of *C. verum*'s bioactive compounds. Among various experimented frequencies, UAE at 40 kHz had the highest content of TPC, TFC, antioxidant properties (DPPH, FRAP, ABTS, and ORAC), and major bioactive compounds (HPLC) followed by UAE at 59 kHz, UAE at 25 kHz, and conventional extraction, respectively. Results presented in this study regarding TPC, TFC, ABTS, DPPH, ORAC, and FRAP in ultrasound extracts of *C. verum* were higher than that reported by Dudonne et al. [36]. This may be due to the difference of extraction methods employed. Herein, we used varied-frequency ultrasound for extraction, while Dudonne et al. [36] extracted *C. verum* using conventional extraction. Moreover, 6-methoxy-7 hydroxycoumarin (Scopoletin), o-coumaric acid, coumarin, cinnamic acid, cinnamaldehyde, and cinnamyl alcohol were also quantified in conventional and ultrasound extracts. Results revealed that the contents of these compounds were higher in ultrasonically extracted *C. verum* extracts as compared with that in conventional extracts. In UAE, phenomena of cavitation result in bubble formation as ultrasound waves travel through the extracting medium. Upon reaching a critical size, these bubbles burst releasing ample amounts of energy. This energy ruptures the cell wall and enhances the penetration power of solvent that is used for extraction. Hence, as compared with conventional extraction, greater penetration of solvent is achieved in a short time, resulting in extraction of more bioactive constituents [46].

Additionally, ultrasound waves disrupt the cellular structure of plant via dissolution of fiber, causing extraction of more phytochemicals from *C. verum* bark in a shorter time as compared with conventional extraction [47]. Because of enhanced disruption of cellular structure of plant, increased interaction time of solvent, and reduced extraction time [47], the extraction of 6-methoxy-7 hydroxycoumarin (Scopoletin), o-coumaric acid, coumarin, cinnamic acid, cinnamaldehyde, and cinnamyl alcohol was greater as compared with conventional extraction. Among varied-frequency ultrasound experimented in this study, maximum content of phenolics (TPC), flavonoids (TFC), antioxidant properties (DPPH, ABTS, FRAP, and ORAC), and chemical compounds (6-methoxy-7 hydroxycoumarin (Scopoletin), o-coumaric acid, coumarin, cinnamic acid, cinnamaldehyde,

and cinnamyl alcohol) were noticed in US at 40 kHz as compared with 25 and 59 kHz. This may be due to poor extractability at 25 kHz and 59 kHz than 40 kHz. The reason for this can be the insufficiency of 25 kHz to extract bioactive compounds from the bark of *C. verum*. On the other hand, maximum frequency experimented (59 kHz) in this study also resulted low extraction than 40 kHz owing to the collapse of the formed bubbles, resulting in reduced formation and power of cavitation in extracting solvent [48, 49]. Similar trend in terms of reduced extraction of compounds at elevated frequencies was reported by Altemimi et al. [50].

In the current study, the highest TPC, TFC, and antioxidant potential was noticed in UAE at 40 kHz followed by UAE at 59 kHz, UAE at 25 kHz, and conventional extraction, respectively. Literature reveals that plant extracts having increased content of TPC and TFC also shows elevated antioxidant properties [51]. Similar trend can be observed from the results of our current study, where maximum antioxidant properties were noticed in extracts with the highest TPC and TFC. Additionally, the quality of plant extracts is determined by antioxidant properties associated with them. The factors regulating the antioxidant properties are mainly harvesting time, maturity index, plant species, extracting conditions, and geological origin [52]. In extraction conditions, other than choice of extraction procedure, the most important features influencing the content of bioactive compounds are processing conditions. In UAE, the type of solvent, extraction temperature, extraction time, and ultrasound power were constant; hence, the only factor affecting the quality of extract was ultrasound frequency.

The results of our study contrast with earlier findings of Phu et al. [39], who used n-hexane as an extracting solvent for UAE of essential oils from *C. verum* bark. On the contrary, in our current study, we employed methanol as an extracting solvent for recovery of polar phytochemicals by following the protocols adopted by Ding et al. [37]. This helped us quantify scopoletin (S), o-coumaric acid (o-CA), coumarin (C), cinnamic acid (CA), cinnamaldehyde (CmA), and cinnamyl alcohol (CyA), resulting in quantification of mainly polar bioactive constituents. Moreover, Phu et al. [39] demonstrated an effective approach to extract nonpolar compounds, while our method is more targeted towards polar compounds having higher antioxidant characteristics. Although solvents used in both studies have toxicity concern, these studies were intended for analytical purposes rather than food consumption. Therefore, in future food application studies, ethanol may be used as an extracting solvent. Likewise, even though current study emphasizes a comparison of ultrasound frequencies with conventional extraction method, an optimization study for other extraction variables (extraction temperature, time, and solvent concentration) were not conducted herein and therefore remains a limitation. On the other hand, even though, the Folin-Ciocalteu analysis is the commonly employed test to determine TPC, it may interact with other reducing constituents that results in overestimations. Therefore, to improve the selectivity and accuracy of results, polyvinylpyrrolidone (PVPP) test may be used in future studies. Keeping in view the limitations, further studies must be conducted

including optimization of other extraction parameters, use of greener solvents, PVPP analysis, and use of diverse plant material for better extraction efficiency and validation.

5. Conclusions

In a nutshell, the results presented in this study signify the use of ultrasound as an alternative technique for efficient extraction of bioactive components from *C. verum* bark. In this study, UAE at three different frequencies (25, 40, and 59 kHz) were used for extraction of phenolic compounds. Here, we demonstrated that UAE at 40 kHz was the most effective extraction method, yielding the TPC, TFC, and antioxidant properties. Furthermore, the noteworthy antioxidant properties in UAE at 40 kHz was due to the occurrence of maximum content of six major biologically active components (6-methoxy-7 hydroxycoumarin (Scopoletin), o-coumaric acid, coumarin, cinnamic acid, cinnamaldehyde, and cinnamyl alcohol). Conclusively, results of our current study highlight the use of ultrasound as a sustainable extraction procedure in enhancing the extracted content of chemical constituents from plant matrix. However, current study lacked optimization of other extracting parameters like solvent ratio, temperature and time that may also affect the extraction efficiency. Moreover, methanol was used in this study for analytical purposes, therefore limiting the direct use in food application. Hence, future research may be designed in a way that it covers optimization of other variables and employ greener solvents to validate the procedure at industrial scale.

Data Availability Statement

Data presented and analyzed in present study are available on request from corresponding authors.

Ethics Statement

The authors have nothing to report.

Disclosure

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Savaiba Khattak: conceptualization, methodology, investigation, data curation, formal analysis, writing – original draft. **Anees Ahmed Khalil:** conceptualization, methodology, data curation, writing – original draft, writing – review and editing, supervision. **Muneeb Khan:** data curation, writing – review and editing. **Muhammad Abid:** writing – review and editing. **Gholamreza Abdi:** writing – review and editing, supervision. **Rana Muhammad Aadil:** methodology, investigation, writing – review and editing, supervision.

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