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Original Article

Extraction and quantification of polyphenols from kinnow (*Citrus reticulata* L.) peel using ultrasound and maceration techniques



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ABSTRACT

An investigation was carried out to extract polyphenols from the peel of kinnow (*Citrus reticulata* L.) by maceration and ultrasound-assisted extraction (UAE) techniques. The antioxidant potential of these polyphenols was evaluated using ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and superoxide radical scavenging assays; and their antimicrobial activity was assessed against bacterial strains *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella typhimurium*. The highest extraction yield was obtained through the solvent ethanol at 80% concentration level, whereas UAE was a more efficient technique and yielded comparatively higher polyphenol contents than maceration. Maximum polyphenols were extracted with 80% methanol [32.48 mg gallic acid equivalent (GAE)/g extract] using UAE, whereas minimum phenolics (8.64 mg GAE/g extract) were obtained with 80% ethyl acetate through the maceration technique. Elevated antioxidant activity of kinnow peel extracts was exhibited in three antioxidant assays, where 80% methanolic extracts showed the highest antioxidant activity (27.67 ± 1.11 mM/100 g for FRAP) and the highest scavenging activity, $72.83 \pm 0.65\%$ and $64.80 \pm 0.91\%$ for DPPH and superoxide anion radical assays, respectively. Strong correlations between total polyphenols and antioxidant activity were recorded. Eleven phenolic compounds—including five phenolic acids and six flavonoids—were identified and quantified by high performance liquid chromatography. Ferulic acid and hesperidin were the most abundant compounds whereas caffeic acid was the least abundant phenolic compound in kinnow peel extracts. Maximum inhibition zone was recorded against *S. aureus* (16.00 ± 0.58 mm) whereas minimum inhibition zone was noted against *S. typhimurium* (9.00 ± 1.16 mm). It was concluded that kinnow mandarin peels, being a potential source of phenolic compounds with antioxidant and antimicrobial properties, may be used as an ingredient for the preparation of functional foods.

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1. Introduction

Polyphenols are natural antioxidants in plants, especially in fruits and vegetables, which have a vital role in human health because of their free radical scavenging activity, antioxidant enzyme cofactors, as well as chelation of pro-oxidant metal ions in the body [1,2]. Epidemiological studies have reported a positive correlation between fruit and vegetable intake and a decrease in the rate of cardiovascular disease, aging, certain cancers, and other degenerative diseases related to oxidative stress, which is attributed to the antioxidant activity of phenolic compounds in fruits and vegetables [3,4].

During the industrial processing of fruits, large quantities of agroindustrial wastes such as peels, seeds, stones, and other residues are produced. The fruit processing wastes contain valuable nutrients and biomass, which may be converted into value-added by-product fruit wastes. In particular, peels have a comparatively higher concentration of phenolic compounds and thus have more antioxidant potential than fruit pulps [5–7].

Citrus is one of the major fruit crops of Pakistan, and comprises kinnow, orange, grapefruit, lemon, lime, sweet orange, etc. The annual production of citrus is 2.33 million tons, of which about 90% is kinnow mandarin [8]. Kinnow mandarin (*Citrus reticulata* L.) peel is about 35–40% of the fruit weight and is the major waste component after processing. High disposal costs of waste have prompted researchers to explore the potential benefits of wastes as well as minimize their environmental hazards [9]. Currently, only a fraction of total peel residue mass is being utilized as beverage bases, marmalades, and candied peel. However, citrus peel is the richest source of bioactive phenolic compounds, especially flavonoids, with comparatively higher polyphenol content compared with the edible parts. The flavonoids present in citrus consist of flavones, isoflavones, flavonones, flavonols, and anthocyanidins [10]. The beneficial effects of citrus peel against certain degenerative diseases (e.g., coronary heart disease) as an antiinflammatory and anticarcinogenic agent have been observed [11].

Solvent extraction is generally used for the preparation of plant material extracts because of its wide applicability, efficiency, and ease of use. Most common organic solvents used for the extraction of phenolic compounds include methanol, ethanol, acetone, and ethyl acetate [12]. Conventional solid–liquid extraction techniques such as maceration are mostly used for obtaining bioactive compound extracts from plant material [13]. However, conventional solvent extraction processes have certain limitations such as high extraction temperature, lower efficiency, low extraction yield, use of large quantity of solvents, mass transfer resistance, and health hazards [14,15]. Ultrasound-assisted extraction (UAE) of polyphenols is a nonconventional technique that involves mixing the sample with organic solvent in a flask or beaker and placing it in an ultrasonic bath with preset time and temperature. Sound waves, which are produced during the process, generate cavitation and rupture sample cell walls, leading to extraction of phenolic compounds from the sample to the solvent medium [16]. Generally, the UAE process duration is less than 1

hour, but the extraction yield is 6% to 35% higher than that obtained using traditional extraction techniques with longer extraction time of 12 or more hours [7,17]. During a study on orange peel polyphenols, Khan et al [18] compared the conventional solvent extraction process with UAE using the 80% ethanol solvent. Significantly high extraction yield and polyphenols flavanone concentration at an ultrasound frequency 25 of kHz and 15 minutes of treatment time was observed as compared to conventional extraction (40°C for 60 minutes). Similarly, Pan et al [19] studied pomegranate peel polyphenols and reported that for the extraction of bioactive compounds, 20 to 100 kHz ultrasonic radiations was effective and could be efficiently used because of high reproducibility, low energy and solvent consumption, and the low temperature used, and thus lower the loss of phenolic compounds.

Antioxidant activity determination of polyphenols *in vitro* is generally carried out using various assays such as ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide radical scavenging assay, trolox equivalent antioxidant capacity, and oxygen radical absorbance capacity. However, DPPH radical scavenging assay is the most popular and widely used technique to evaluate antioxidant capacity [20,21].

Plants generate a variety of secondary metabolites as part of their defense system during growth. These secondary metabolites or phytochemicals have strong inhibitory activity against microorganisms such as bacteria and fungi [22]. Bacteria as well as fungal infections pose a big threat to mankind, and indiscriminate use of antimicrobial drugs has caused resistance in microbes. Because they have the least antibiotic-related side effects and better activity against drug-resistant strains, researchers have focused their attention toward phytochemicals [23]. Phytochemicals abundant in plants include phenolic acids, flavonoids, tannins, and alkaloids. The antimicrobial characteristics of certain polyphenol classes have been investigated to develop novel therapies for the treatment of different microbial infections [24,25]. Agroindustrial wastes were studied for their potential antimicrobial activity by different researchers such as lemon peels [26], pomegranate peels [27], grape marcs [28], and grape seeds [29].

Keeping in view the abovementioned facts, a research study was designed to optimize extraction conditions for polyphenols from kinnow peels, determination of antioxidant and antimicrobial activity of phenolic compounds in kinnow peels.

2. Materials and methods

2.1. Plant material

Kinnow mandarin (*C. reticulata* L.) were procured from a fruit market in Islamabad and taken to the Food Science and Product Development Institute research laboratory, National Agricultural Research Center. Fruits were thoroughly washed under tap water to remove dirt, dust, microflora, and pesticide residue on the surface. Peeling of kinnow mandarin was carried out; the, the peels were further cut into small pieces using a stainless

steel knife and oven-dried at 50°C for 48 hours in hot air oven until moisture content fell below 10%. Dried peels were ground to fine powder through the cyclotec sample mill with sieve size 0.5 mm. Kinnow mandarin peel powder was packed in airtight polyethylene zip bags and stored at refrigeration temperature.

2.2. Proximate analysis of peel powder

Kinnow peel powder was analyzed for moisture, ash, crude protein, crude protein, crude fat, and crude fiber according to the standard methods of the Association of Analytical Communities [30]. Available carbohydrate in peel powder was estimated by difference [100 – (% moisture + % ash + % crude protein + % crude fat + % crude fiber)].

2.3. Extraction of polyphenols

Maceration and UAE procedures were used for polyphenol extraction from kinnow mandarin peel powders.

2.4. Maceration

Kinnow mandarin peel powders were subjected to extraction through the maceration technique as described by Elfalleh et al [31] with slight modifications. Preliminary studies were performed to evaluate an optimal sample/solvent ratio (1:10, 1:15, 1:20) and extraction temperature (30°C and 40°C). After the preliminary studies, extraction was carried out using different solvents—ethanol, methanol, acetone, and ethyl acetate—at three solvent concentrations (50%, 80%, 100%) with a 1:15 sample/solvent ratio and extraction temperature of 40°C. Briefly, 5-g kinnow peel powder samples were extracted by specific solvent, concentration level, extraction temperature, and sample/solvent ratio in a shaking water bath (Tecator 1024; Tecator AB, Höganäs, Sweden) for 20 hours. The extracts were filtered through Whatman filter paper 1 and centrifuged (Beckman J2-21; Beckman Coulter, Fullerton, CA, USA) at 5000 rpm for 10 minutes. The supernatant was collected, and the solvent was evaporated with a rotary evaporator (BUCHI Rotavapor, Flawil, Switzerland) under vacuum at 45°C to obtain the extract, which was further filtered through 0.45- μ m membrane filter, then collected in amber glass bottles and stored at refrigeration temperature.

2.5. Ultrasound-assisted extraction

The extraction of polyphenols from kinnow mandarin peel powder was conducted using the UAE technique as described by Bimakr et al [32] with slight modifications. Preliminary studies were carried out to determine the optimal sample/solvent ratio (1:10, 1:15, 1:20), extraction temperature (35°C, 45°C, 55°C), and extraction time (40 minutes, 50 minutes, 60 minutes, and 70 minutes). After the preliminary studies, 5-g kinnow peel powder samples were extracted by solvents ethanol and methanol at 50%, 80%, 100% concentration levels under optimal extraction conditions: sample/solvent ratio, 1:20; extraction temperature, 45°C; extraction time, 60 minutes in a sonicator (Transsonic 700; Elma, Wetzikon, Switzerland) at 35 kHz frequency. The extracts were subjected to filtration, centrifugation, solvent vacuum evaporation, microfiltration,

collection in amber glass bottles, and refrigeration (storage) in a similar manner with maceration extracts.

2.6. Yield (%) of peel extracts

The percent yield of kinnow peel extracts through maceration and UAE was assessed by dividing the weight of the extract with the sample weight and multiplying by 100.

2.7. Total polyphenols determination

The total polyphenol content of kinnow mandarin peel extracts was measured using the Folin–Ciocalteu method as described by Singleton et al [33]. Methanolic solutions of kinnow peel extracts (10 mg/mL) were prepared for the analysis. Briefly, 0.5 mL methanolic extract solution was mixed with 2.5 mL of 10% Folin–Ciocalteu reagent dissolved in distilled water and 2.5 mL 7.5% sodium carbonate. The blank contained 0.5 mL methanol, 2.5 mL Folin–Ciocalteu reagent (10 times diluted), and 2.5 mL of 7.5% sodium carbonate. Then the samples were incubated at 25°C for 30 minutes for the development of a blue color. The absorbance was measured at 765 nm with a UV–VIS Spectrophotometer (Agilent 8453; Santa Clara, California, USA). A similar procedure was carried out for gallic acid standard solution, and the calibration curve was prepared from various concentrations of gallic acid. The total polyphenol content was expressed as mg gallic acid equivalent (GAE)/g extract.

2.8. Antioxidant activity evaluation

2.8.1. FRAP assay

The FRAP assay was carried using the procedure described by Benzie and Strains [34] with several modifications. The FRAP reagent was prepared by mixing 25 mL of 0.3M acetate buffer (pH 3.6) with 2.5 mL 2,4,6-Tripyridyl-s-Triazine (TPTZ) solution (0.01M) and 2.5 mL of FeCl₃·6H₂O (0.02M). A 200- μ L diluted sample was added to 1.5 mL FRAP reagent and warmed at 37°C for 10 minutes. The absorbance was measured at 593 nm, and the antioxidant activity of the sample was expressed as millimoles per 100 g of extract.

2.8.2. DPPH radical scavenging assay

The antioxidant activity of kinnow mandarin peel extracts was measured using the DPPH (1,1-diphenyl-2-picryl-hydrazyl) assay according to the method described by Brand-William et al [35] with slight modifications. Briefly, 24 mg DPPH was dissolved in 100 mL methanol to prepare a stock solution. The working standards were prepared by diluting DPPH stock solution with methanol to obtain about 0.98 (\pm 0.02) absorbance at 517 nm. Then, 3 mL of the solution was mixed with 100 μ L of samples at different concentrations (25–400 μ g/mL), shaken well, incubated in the dark at room temperature for 15 minutes, and absorbance was measured at 517 nm. A parallel control (without extract) and standard ascorbic acid were also analyzed in a similar manner. The scavenging activity was calculated based on the DPPH radical percentage scavenged.

$$\% \text{ Inhibition of DPPH radical} = \frac{A_c - A_s}{A_c} \times 100,$$

where A_c is the absorbance of control and A_s is the absorbance of the sample.

2.8.3. Superoxide radical scavenging power assay

The antioxidant activity of kinnow peel extracts was determined by superoxide radical scavenging assay in accordance with the procedure described by Vaidya et al [36]. Initially, 1 mL kinnow peel extract at different concentrations (25–400 $\mu\text{g/mL}$) was added to 1 mL sodium carbonate (5%), 0.3 mL EDTA (0.5%), and 0.4 mL nitroblue tetrazolium (150 μM). After mixing all the reagents, absorbance was measured immediately at 560 nm. The reaction was initiated by the addition of 0.4 mL hydroxylamine hydrochloride and incubated at 25°C for 5 minutes. The nitroblue tetrazolium (NBT) reduction was determined with a spectrophotometer at 560 nm. A parallel control (without extract) and standard ascorbic acid were also analyzed in a similar manner. The % scavenging activity was calculated as follows:

$$\% \text{ Inhibition of superoxide radical} = [1 - A_1/A_0] \times 100,$$

where A_1 is the absorbance of extract sample and A_0 is the absorbance of control.

2.9. High performance liquid chromatography analysis of phenolic compounds

Identification and quantification of phenolic acids and flavonoids in the extracts were determined with high performance liquid chromatography (HPLC) according to the method described by Salvador et al [37] with slight modifications. Kinnow mandarin peel extract samples filtered through a 0.45- μm membrane filter were injected into the HPLC system, which consisted of a Perkin Elmer HPLC equipped with Binary LC pump 250, an LC295 UV/VIS detector, and a reversed phase C18-WP.100 column (CNW Technologies, Dusseldorf, Germany) with internal dimensions of 4.6 mm \times 250 mm, 5 μm . The mobile phase consisted of a linear gradient with a combination of solvent A (acetonitrile) and solvent B (distilled water/acetic acid, 99:1, v/v, pH 2.30 \pm 0.1). The following gradient program was used for the separation of flavonoids and phenolic acids: 20% A (5 min), 80% A (10 min), 20% A (5 min). The analyses were conducted at a flow rate of 1 mL/min with the UV detector set at 280 nm for phenolic acids and 370 nm for flavonoids and a sample injection volume of 20 μL . The analytes were identified by comparing the retention times and spike samples with polyphenol standards (gallic acid \geq 99%, coumaric acid \geq 98%, chlorogenic acid \geq 95%, caffeic \geq 98%, ferulic acid \geq 99%, catechin \geq 98%, epicatechin \geq 98%, hesperdin \geq 97%, naringenin \geq 95%, quercetin \geq 95%, kaempferol \geq 90%; Sigma Aldrich, Saint-Quentin-Fallavier, France) and subsequent quantification of phenolic compounds were determined.

2.10. Antimicrobial activity determination

The antimicrobial activity of kinnow mandarin peel extracts was determined using the disk diffusion method [38,39]. Two Gram-positive bacterial strains (*Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 10876) and one Gram-negative

strain (*Salmonella typhimurium* ATCC 14028) were selected for the study of the antimicrobial activity of kinnow peel extracts.

For the preparation of media, nutrient agar was dissolved in distilled water and the pH was adjusted to 7. Sterilization of media was carried out in the autoclave at 121°C for 15 minutes, and the media were cooled at room temperature after sterilization. The petri plates without any contamination were selected for further investigations. Sterile paper disks (6 mm diameter) were placed on the agar medium. Then, 10 μL extract sample of various concentrations (25 mg/mL, 50 mg/mL, 100 mg/mL, 200 mg/mL extract) was applied on sterile disks and allowed to dry. For comparison, the antibiotic chloramphenicol was used as a standard. Bacterial cultures were injected to sterilize petri plates. The petri plates were incubated at 37°C for 24 hours, after which the zones of growth inhibition (mm) around the disks were measured.

2.11. Statistical analysis

Data were statistically analyzed using analysis of variance to determine the significance level. The least square design (LSD) test was used to calculate the least significant difference among means. Minitab software was used for conducting statistical analysis of data.

3. Results and discussion

3.1. Extraction yield

The percent yield of kinnow mandarin peel extracts through UAE and maceration techniques at different solvent concentration levels reveals that the highest extraction yield was obtained with solvent ethanol either through maceration or UAE (Figure 1). In the case of the maceration technique, extraction with 80% ethanol resulted in the highest yield (18.46%) followed by 50% ethanol extraction (15.64%), whereas the lowest extraction yield (5.12%) was recorded in samples extracted with 100% ethyl acetate. As regards UAE, extraction with 80% ethanol led to a comparatively higher yield (19.24%) than samples extracted with solvent methanol. Extraction with 100% methanol solvent concentration resulted in the lowest extraction yield (13.84%). Statistically, solvent concentration levels were significantly different from each other for solvent methanol and ethanol used in the UAE method, whereas there were nonsignificant difference between concentration levels 50% and 80% for solvent ethyl acetate as well as 80% and 100% for acetone in the maceration technique. However, the solvent concentration level of 80% was more effective than 50% or 100% solvent concentration of all solvents used during maceration and UAE. Overall, UAE had a comparatively higher extraction yield at all solvent concentration levels compared with the maceration technique. Variations in extraction yield among the various solvents used may be attributable to the different polarities of solvents. Sultana et al [40] investigated various agro wastes and observed a polyphenol extraction yield of 21.5% from citrus peels with 80% methanol solvent. Similarly, Hegazy and Ibrahim [41] reported the orange peel extract yield within the range of 8.27% from solvent hexane to 28.32% from methanol.

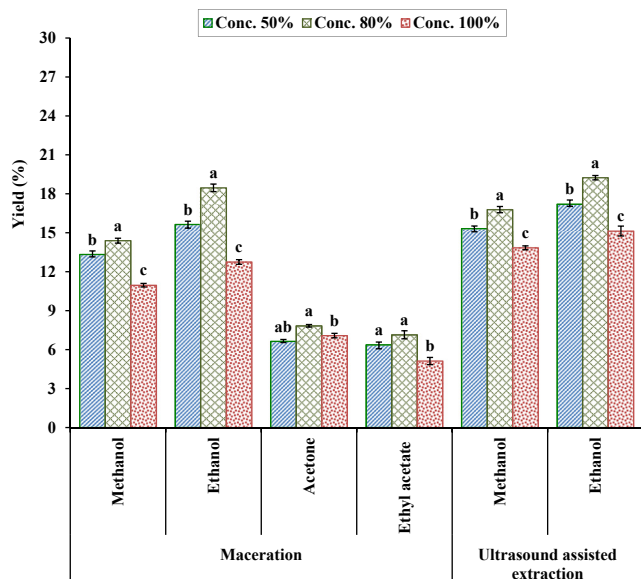


Figure 1 – Yield (%) of kinnow mandarin peel extracts by maceration and ultrasound-assisted extraction. Values are presented as mean \pm standard error of triplicate analyses. Same alphabetical letters denote nonsignificant difference at $p < 0.05$.

The yield of phenolic compounds from plants is associated with the polarity, solubility, as well as certain extraction parameters such as nature of solvent, solvent concentration, extraction temperature, and time [42,43].

3.2. Total polyphenols content

Maceration and UAE methods were applied for the extraction of polyphenols, which were then determined using the Folin–Ciocalteu reagent assay. The total polyphenol content of kinnow mandarin peel extracts shows that maceration (Figure 2) was comparatively less efficient than the UAE (Figure 3) technique, which yielded higher polyphenol content. As regards maceration extraction, methanol was the most effective solvent followed by ethanol, whereas ethyl acetate had the lowest polyphenol extraction rate. The highest total polyphenol contents (28.40 ± 0.33 mg GAE/g extract) were extracted with solvent methanol at 80% concentration level, whereas lowest polyphenol contents (8.64 ± 0.20 mg GAE/g extract) were obtained with 80% ethyl acetate. Results of the LSD test reveal that solvent concentration levels were significantly different from each other for methanol and ethanol used, whereas there were nonsignificant differences between concentration levels 50% and 80% for solvent acetone and ethyl acetate when the maceration technique was used. Al-Juhaimi [44] extracted polyphenols through the maceration technique from the peel and pulp of mandarin, lemon, and orange using 80% ethanol as solvent at 70°C for 3 hours, and phenolic compounds of extracts were evaluated with the Folin–Ciocalteu reagent assay. They observed that mandarin, orange, and lemon peels contained 169.54 mg GAE/100 g, 178.90 mg GAE/100 g, and 61.22 mg GAE/100 g total phenolics,

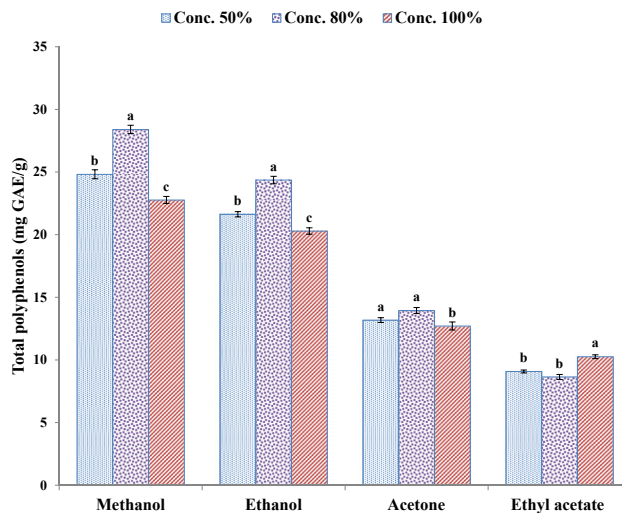


Figure 2 – Total polyphenol content (mg GAE/g extract) of kinnow peel extracts by maceration technique. Values are presented as mean \pm standard error of triplicate analyses. Data with different letters denote significant difference at $p < 0.05$. GAE = gallic acid equivalent.

which were higher than the amount of phenolic compounds extracted from pulp.

In the case of UAE, maximum polyphenols were extracted with 80% methanol (32.48 ± 0.36 mg GAE/g extract) whereas 100% ethanolic extracts had minimum phenolics (24.39 ± 0.28 mg GAE/g extract). The LSD test result reveals that solvent concentration levels had a significant effect on phenolic extraction and were significantly different from each other at all concentration levels of both solvents. Because of

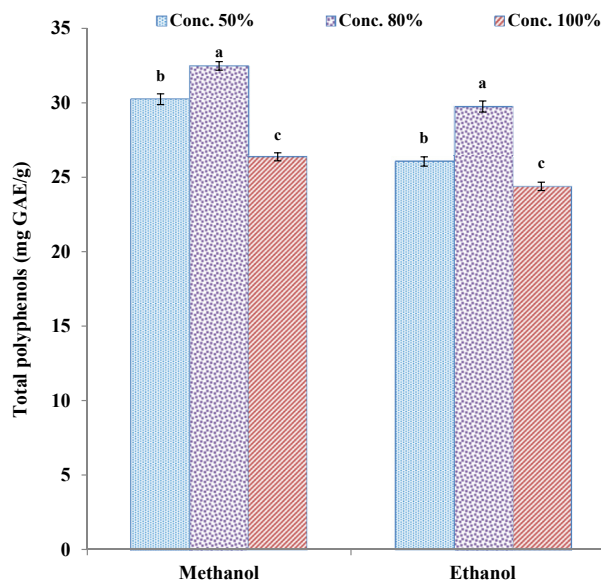


Figure 3 – Total polyphenol content (mg GAE/g extract) of kinnow peel extracts by ultrasound assisted extraction. Values are presented as mean \pm standard error of triplicate analyses. Different alphabetical letters denote significant difference at $p < 0.05$. GAE = gallic acid equivalent.

the ultrasonic cavitation phenomenon of UAE, the cavitation generates currents in the solvent, which in turn increases the mass transfer rate between the sample material and the solvent medium [16], causing mechanical effects on samples cell walls that result in cell disruption and particle breakdown [45]. The advantage of UAE over the maceration technique is the comparatively higher extraction of polyphenols in a shorter time, thus saving energy inputs [46]. Furthermore, phenolic compounds being thermosensitive remained stable during UAE as compared to Soxhlet and other conventional techniques where elevated temperature are used [47]. The total polyphenol content of kinnow peel extracts obtained by UAE was 14.37% higher than the polyphenols extracted through the maceration technique at 80% methanol solvent concentration. These results are in agreement with the findings of Petigny et al [48], who used maceration and UAE methods for the extraction of polyphenols from boldo leaves and observed 20% more polyphenolic content extracted through UAE compared with maceration. Likewise, UAE and maceration techniques were compared by Quiroz-Reyes et al [49] for the extraction of phenolic compounds from cocoa beans and reported 50% higher polyphenol content extracted by UAE compared with maceration.

Extraction of polyphenols also depends on the type of solvent used. Chan et al [50] compared various solvents such as ethanol, acetone, and methanol for extraction of polyphenols from limau purut (*Citrus hystrix*) peel and concluded that aqueous acetone was slightly more efficient than aqueous ethanol and aqueous methanol water with the following extraction conditions: 60% solvent; temperature, 25°C; extraction time, 3 hours. Similarly, the efficiency of various solvents such as ethanol, methanol, acetone, dichloromethane, ethyl acetate, and hexane were assessed for the extraction of polyphenols from orange peel [41]. It was observed that there was variation in total polyphenol content among different solvent extracts with ethanolic extract having the highest total polyphenols (169.56 mg/g) whereas hexane extract contained the lowest total polyphenol content (63.20 mg/g). The total polyphenols of each solvent at absolute concentration level were lowest for both extraction methods, which established the findings of Chan et al [50] that absolute solvent could not ensure fair extraction of polyphenols than aqueous solvents. Selection of the appropriate extraction solvent is vital for complex food matrices as it will estimate the type and quantity of polyphenols being extracted. Variations in extracted polyphenol content depend on the polarities of the solvents used as well as their concentration level, either aqueous or absolute. In general, aqueous alcohols such as methanol and ethanol are

used in extraction of phenolic compounds from plant materials [51]. Solvent ethanol categorized under GRAS (Generally Recognized as Safe) is preferred because of its application in the food system. Ethanol enhances the solubility of a solute, whereas water accelerates its desorption from the sample matrix [52]. The low solubility of phenolic compounds in absolute solvents may be attributable to the strong hydrogen bonding between protein and polyphenols. However, the solubility increases upon addition of water to organic solvents that weakens the hydrogen bonds [53]. In a related study, Nepote et al [54] investigated the phenolic content of peanut skins with different concentrations of ethanol and reported that 50% ethanol led to the highest polyphenol content, which decreased with the increase in ethanol concentration above 70%.

3.3. Antioxidant activity

3.3.1. FRAP assay

The antioxidant power of a sample extract corresponds to its reducing capacity to transfer electrons to a FRAP reagent. The FRAP data (Table 1) indicate that kinnow mandarin peel extracts exhibited high antioxidant activity extracted with methanol as well as ethanol solvents. However, peel samples extracted with methanol had significantly higher antioxidant activity than samples extracted with ethanol. As regards the solvent concentration level, 80% methanolic extracts exhibited highest antioxidant activity (27.67 ± 1.11 mM/100 g) followed by 80% ethanolic extracts (25.82 ± 0.67 mM/100 g), whereas polyphenols extracted with 50% ethanol had the least antioxidant activity (21.29 ± 0.70 mM/100 g). The LSD test results show that there were nonsignificant differences between 50% and 100% ethanolic extracts, but significant difference from 80% ethanolic extracts. However, methanolic extracts at different concentration levels were significantly different from each other. While investigating different fruit wastes for antioxidant activity, Farha et al [55] observed that 50% methanolic extracts of sweet lime (*Citrus limetta*) had a FRAP value of 7.48 mmol Fe²⁺/mL, which was considered a medium antioxidant activity. During a related study on various fruit peels' antioxidant activity, Zulkifli et al [56] reported a FRAP value of 20.03 ± 1.46 mM/100 g for Navel orange (*Citrus sinensis*) peel using water extraction. Similarly, Oikeh et al [57] evaluated the *in vitro* antioxidant activity of sweet orange (*C. sinensis*) wastes and found that 70% ethanolic extract of flavedo had maximum FRAP value (800.30 ± 1.53 μmol Fe²⁺/g extract) whereas the absolute ethanolic sweet orange seed extracts had minimum FRAP value (329 ± 1.53 μmol Fe²⁺/g extract). Variations in the FRAP activity of citrus peel among different studies may be

Table 1 – Antioxidant activity of kinnow mandarin peel extracts.

Antioxidant assays	Methanol			Ethanol		
	100%	80%	50%	100%	80%	50%
FRAP	21.95 ± 1.44^c	27.67 ± 1.91^a	24.08 ± 1.59^b	$22.53 \pm 1.32^{b,c}$	$25.82 \pm 1.15^{a,b}$	21.29 ± 1.66^c
DPPH	55.61 ± 1.69^d	72.83 ± 1.12^a	60.67 ± 1.24^c	57.18 ± 1.49^d	$69.74 \pm 1.97^{a,b}$	56.52 ± 0.92^d
Super oxide	56.86 ± 1.37^c	64.80 ± 1.57^a	59.19 ± 0.83^b	55.28 ± 0.99^c	61.37 ± 1.63^b	54.06 ± 1.11^d

Data sharing similar letters in a row are statistically nonsignificant ($p > 0.05$).

All values represent the mean of three replications \pm standard error ($n = 3$).

DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = ferric reducing antioxidant power.

influenced by type of citrus variety, solvent used, as well as solvent concentration.

3.3.2. DPPH radical scavenging activity

DPPH assay has been used widely and is a popular technique to assess the free radical scavenging activity of different plant extracts. It is a stable free radical that dissolves in either ethanol or methanol, and DPPH free radical reduction is determined by the decrease in its absorption at 517 nm when the color of the DPPH assay solution changes from purple to light yellow. The scavenging potential of plant extract antioxidants corresponds to the degree of the discoloration [58].

The effect of different solvents and their concentration levels on DPPH radical scavenging activity of kinnow mandarin peel extracts (Table 1) reveals high antioxidant activity of all sample extracts. However, the highest scavenging activity ($72.83 \pm 0.65\%$) was exhibited by samples extracted with solvent methanol at 80% concentration level followed by 80% ethanolic extract ($69.74 \pm 1.14\%$), whereas samples extracted with 100% methanol had the lowest scavenging activity ($55.61 \pm 0.98\%$). Aqueous solvent extracts had higher inhibitory activity against the DPPH radical as compared to corresponding absolute solvents, which may be attributed to the higher polyphenol content in these extracts. Among solvents, methanol extracted samples exhibited more scavenging activity than samples extracted with ethanol. When compared to standard ascorbic acid, the DPPH radical scavenging activity of kinnow peel extracts was lower ($95.83 \pm 0.75\%$). The extracting solvent effect on DPPH radical scavenging activity was earlier reported by Turkmen et al [59]. During a study on natural antioxidants from citrus mandarin peels, Karsheva et al [60] observed that 50% ethanolic extracts had the highest DPPH radical scavenging activity compared with 20% and 70% ethanolic extracts. Similarly, Do et al [61] investigated the effect of extraction solvent on the antioxidant activity of *Limnophila aromatica* and observed that 100% ethanolic extract exhibited the maximum DPPH radical scavenging activity. Likewise, the peel and pulp of kinnow mandarin, orange, and lemon were assessed for total polyphenols and free radical scavenging activities. It was reported that orange pulp exhibited the highest radical scavenging activity (69.31%) followed by kinnow mandarin peel (68.57%), and lemon peel had the lowest radical-scavenging activity (46.98%). Park et al [62] found that the DPPH radical scavenging activity of orange flesh was higher than that of orange peel and reported that the acetone extract of orange flesh had the highest DPPH radical scavenging activity (compared with ethanolic and methanolic extracts). The IC_{50} value (i.e., the sample concentration required to scavenge 50% free radicals) was lowest in orange flesh acetone extracts (3333.7 $\mu\text{g/mL}$). The IC_{50} value is negatively correlated to antioxidant activity, and the lower the IC_{50} value, the higher the sample antioxidant activity [63]. Similarly, Oikeh et al [57] observed that the IC_{50} value of 70% ethanolic extract of sweet orange (*C. sinensis*) seeds was lowest (0.18 mg/mL) and hence had more radical scavenging activity than albedo and flavedo extracts.

3.3.3. Superoxide radical scavenging power assay

Although considered a weak oxidant, superoxide anion radical may lead to the generation of dangerous and powerful

hydroxyl radicals and singlet oxygen, which are responsible for oxidative stress-related disorders. The antioxidants scavenge the superoxide anion and the percentage scavenging of superoxide anion radical increases with the increase in concentration of antioxidants [64].

The effect of different solvents and their concentration levels on superoxide anion radical scavenging activity of kinnow mandarin peel extracts (Table 1) shows the fairly high antioxidant activity of sample extracts. However, kinnow peel methanolic extracts at 80% concentration level exhibited the highest activity to scavenge superoxide anion radical ($64.80 \pm 0.91\%$) followed by 80% ethanolic extract ($61.37 \pm 0.91\%$), whereas the 50% ethanolic extract had the lowest scavenging activity ($54.06 \pm 0.64\%$). Overall, the superoxide anion radical scavenging activity of kinnow mandarin peels was comparatively lower than that of the standard ascorbic acid ($87.83 \pm 0.92\%$). In general, aqueous solvent extracts demonstrate lower inhibitory activity against superoxide anion radical compared with absolute solvent concentration extracts. The antioxidant activities of the pulp and peel of citrus fruits kinnow, orange, and shaddock were assessed by Mathur et al [65]. It was noted that the ethanolic extract of the peel and pulp of citrus fruits had higher superoxide radical scavenging activity than aqueous and chloroform extracts. Kinnow peel (87%) and shaddock pulp (90%) exhibited the highest scavenging activity. Similarly, Kalpna et al [58] evaluated the antioxidant potential of different fruit and vegetable peels using methanol, acetone, chloroform, and hexane. It was observed that the acetone extract of mango peels had the highest superoxide radical scavenging activity compared with methanolic, chloroform, and hexane extracts as well as other fruit and vegetable extracts. Jahan [66] investigated the superoxide anion radical scavenging activity of different medicinal plants and reported that methanolic extracts had a stronger antioxidant activity compared with water extracts, which might be attributable to the presence of high concentrations of hydrophilic and hydrophobic phenolic compounds.

3.4. HPLC analysis of phenolic compounds

Identification and quantification of phenolic acids and flavonoids in kinnow mandarin peel extracts were determined with HPLC. A total of 11 phenolic compounds—including five phenolic acids and six flavonoids at wavelength 280 nm and 370 nm, respectively—were identified and quantified according to retention time and their peaks' spectral characteristics against those of standards (Table 2). HPLC chromatograms of peel extracts and phenolic standards are presented in Figures 4 and 5. It is evident from the data that kinnow mandarin peel extracts phenolic compounds varied considerably as a function of solvent composition and concentration level. Maximum phenolic compounds were quantified in 80% ethanolic extracts ($371.16 \pm 6.79 \mu\text{g/g}$) followed by 50% methanolic extracts ($350.17 \pm 4.47 \mu\text{g/g}$) whereas minimum phenolic compounds were quantified in 100% ethanolic extracts ($178.75 \pm 2.12 \mu\text{g/g}$) of kinnow mandarin peels. Among the phenolic compounds, ferulic acid and hesperidin were the most abundant in kinnow mandarin peel extracts. Maximum ferulic acid ($102.13 \pm 1.51 \mu\text{g/g}$)

Table 2 – Effect of solvent type and concentration on the phenolic compounds profile in kinnow peel.

Phenolic compounds (µg/g)	Methanol			Ethanol		
	100%	80%	50%	100%	80%	50%
Gallic acid	37.86 ± 1.03 ^c	39.54 ± 1.29 ^c	48.05 ± 0.71 ^b	12.02 ± 0.44 ^e	54.13 ± 1.12 ^a	25.60 ± 0.70 ^d
Chlorogenic acid	18.48 ± 0.41 ^b	12.91 ± 0.47 ^d	22.48 ± 0.85 ^a	17.25 ± 0.64 ^{b,c}	20.52 ± 0.82 ^{a,b}	15.86 ± 0.42 ^c
Ferulic acid	50.16 ± 0.75 ^d	88.41 ± 0.86 ^b	102.13 ± 1.51 ^a	22.37 ± 0.94 ^f	65.21 ± 1.16 ^c	42.56 ± 1.05 ^e
Coumaric acid	17.12 ± 0.34 ^{b,c}	11.23 ± 0.50 ^d	22.51 ± 0.61 ^a	15.93 ± 1.04 ^c	27.29 ± 0.44 ^f	20.18 ± 0.35 ^{a,b}
Caffeic acid	1.28 ± 0.38 ^b	N.D.	N.D.	N.D.	2.43 ± 0.30 ^a	N.D.
Catechins	26.24 ± 0.93 ^d	32.06 ± 0.44 ^c	37.89 ± 0.54 ^b	18.54 ± 0.49 ^e	49.46 ± 1.03 ^a	36.42 ± 0.88 ^b
Epicatechins	20.54 ± 0.53 ^a	17.25 ± 0.63 ^{a,b}	14.46 ± 0.33 ^b	N.D.	18.62 ± 0.54 ^a	7.73 ± 0.60 ^c
Hesperidin	44.38 ± 1.08 ^f	52.14 ± 1.22 ^e	61.02 ± 1.17 ^d	75.66 ± 1.67 ^c	92.94 ± 1.23 ^a	84.41 ± 1.01 ^b
Naringenin	1.97 ± 0.37 ^b	N.D.	3.74 ± 0.45 ^a	N.D.	N.D.	2.52 ± 0.28 ^b
Quercetin	18.44 ± 0.65 ^d	29.78 ± 0.86 ^a	25.71 ± 0.80 ^{b,c}	16.98 ± 0.39 ^d	23.71 ± 0.50 ^c	26.98 ± 0.65 ^b
Kaempferol	12.52 ± 0.32 ^b	13.87 ± 0.54 ^{a,b}	12.18 ± 0.39 ^b	N.D.	16.85 ± 0.41 ^a	14.26 ± 0.66 ^{a,b}
Total	248.99 ± 5.04 ^c	297.19 ± 2.64 ^b	350.17 ± 4.47 ^a	178.75 ± 2.12 ^d	371.16 ± 6.79 ^a	276.52 ± 5.26 ^{b,c}

All values are the mean of three replications.

Means followed by same letters do not differ significantly ($p < 0.05$).

Different superscript letters within same row denote significant difference ($p < 0.05$).

N.D. = not detected.

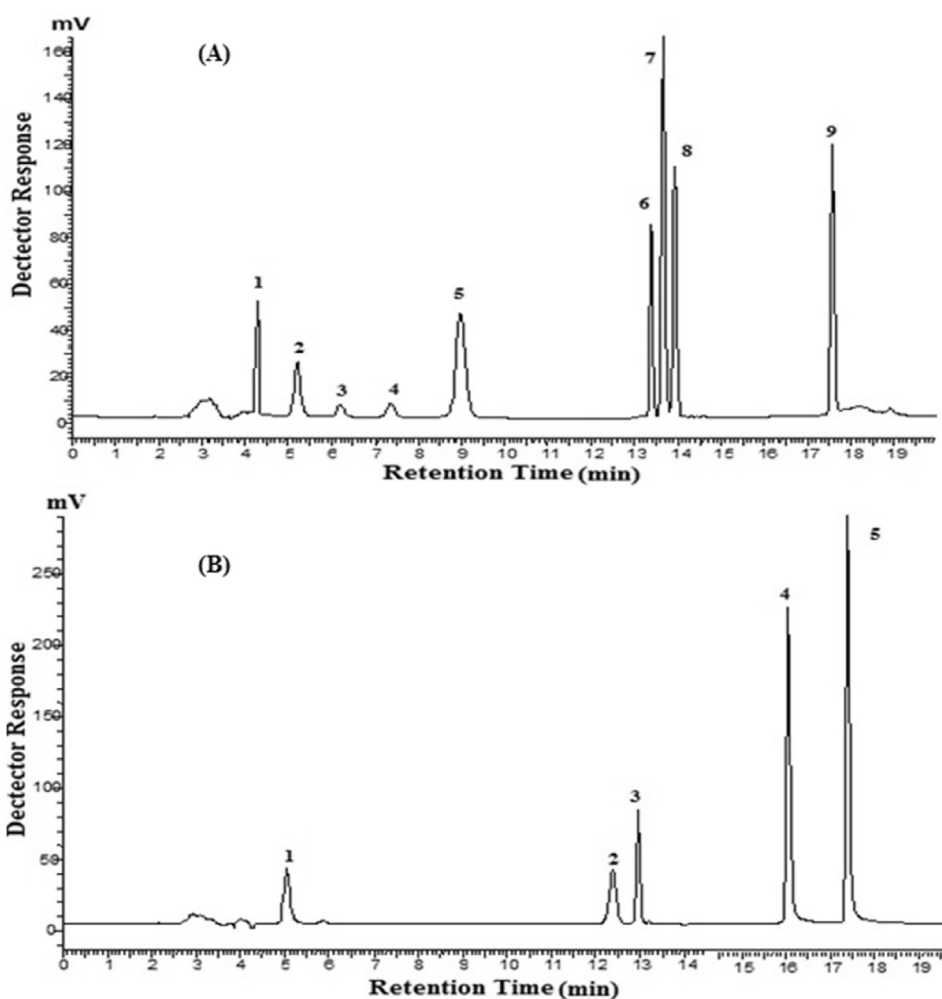


Figure 4 – (A) Typical chromatogram of polyphenols standards (200 µg/mL) at 280 nm. 1 = gallic acid, 2 = chlorogenic acid, 3 = catechin; 4 = epicatechin; 5 = caffeic acid; 6 = hesperidin; 7 = trans-ferulic acid; 8 = coumaric acid; 9 = naringenin. (B) Typical chromatogram of polyphenol standards (200 µg/mL) at 370 nm. 1 = magniferin; 2 = myricetin; 3 = rutin; 4 = quercetin; 5 = kaempferol.

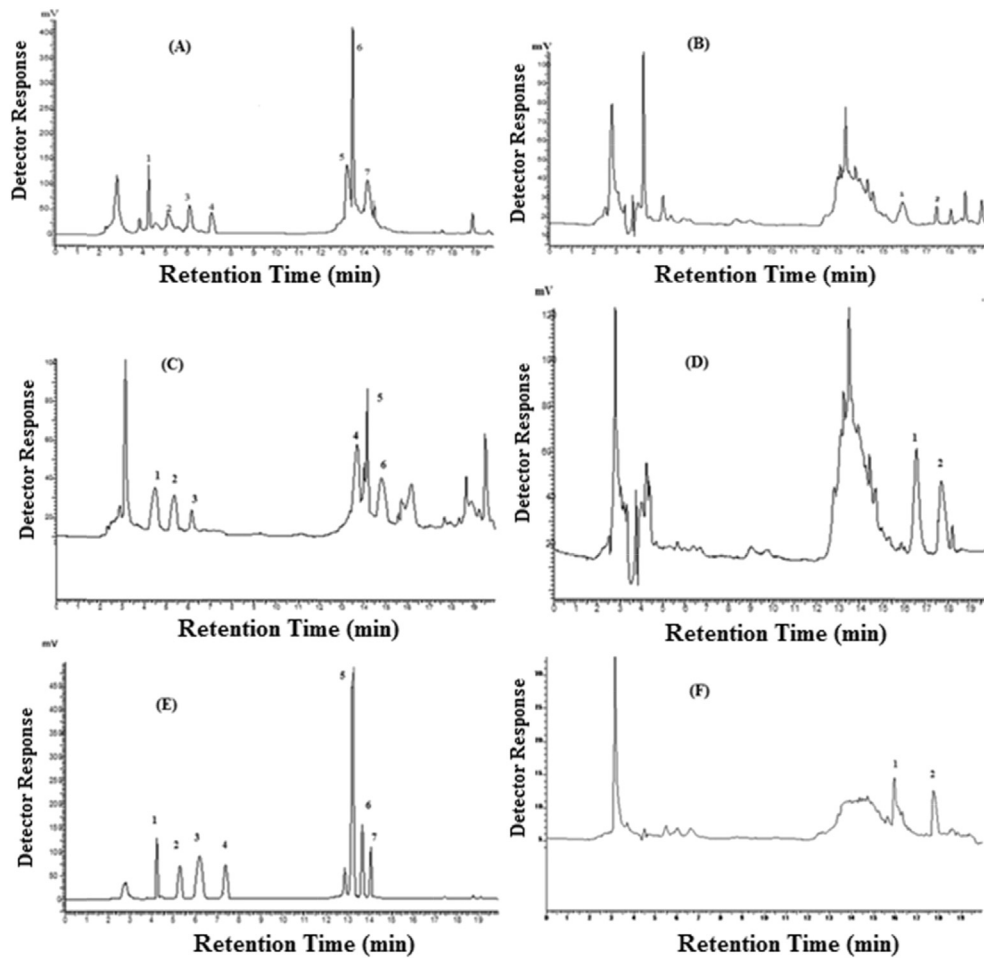


Figure 5 – (A) Typical chromatogram of polyphenols of kinnow mandarin peel 50% methanolic extract at 280 nm, 1 = gallic acid, 2 = chlorogenic acid, 3 = catechin, 4 = epicatechin, 5 = hesperidin, 6 = ferulic acid, 7 = coumaric acid. (B) Typical chromatogram of polyphenols of kinnow mandarin peel 50% methanolic extract at 370 nm, 1 = quercetin, 2 = kaempferol. (C) Typical chromatogram of polyphenols of kinnow mandarin peel 100% methanolic extract at 280 nm, 1 = gallic acid, 2 = chlorogenic acid, 3 = catechin, 4 = hesperidin, 5 = ferulic acid, 6 = coumaric acid. (D) Typical chromatogram of polyphenols of kinnow mandarin peel 100% methanolic extract at 370 nm, 1 = quercetin, 2 = kaempferol. (E) Typical chromatogram of polyphenols of kinnow mandarin peel 80% ethanolic extract at 280 nm, 1 = gallic acid, 2 = chlorogenic acid, 3 = catechin, 4 = epicatechin, 5 = hesperidin, 6 = ferulic acid, 7 = coumaric acid. (F) Typical chromatogram of polyphenols of kinnow mandarin peel 80% ethanolic extract at 370 nm, 1 = quercetin, 2 = kaempferol.

Table 3 – Antimicrobial activity of kinnow mandarin peel extracts.

Extract conc. ($\mu\text{g}/\text{disk}$)	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Salmonella typhimurium</i>	Mean
250	N.D.	N.D.	N.D.	N.D.
500	$8.67 \pm 0.33^{\text{d,e}}$	N.D.	N.D.	$2.89 \pm 0.56^{\text{C}}$
750	$14.00 \pm 1.16^{\text{b,c}}$	$12.67 \pm 1.02^{\text{c}}$	$7.33 \pm 0.96^{\text{e}}$	$11.33 \pm 1.05^{\text{B}}$
1000	$16.00 \pm 0.58^{\text{a}}$	$14.33 \pm 0.88^{\text{b}}$	$9.00 \pm 1.16^{\text{d}}$	$13.11 \pm 0.87^{\text{A}}$

Data sharing similar letters in a row or in a column are statistically nonsignificant ($p > 0.05$). Small letters represent comparison among interaction means and capital letters are used for overall mean.

All values represent the mean of inhibition zone (mm) \pm standard error ($n = 3$).

N.D. = not detected.

and hesperidin ($92.94 \pm 1.23 \mu\text{g/g}$) were determined in 50% methanolic and 80% ethanolic extracts, respectively. Gallic acid and catechin were the other phenolic compounds present in high concentration, whereas caffeic acid and naringenin were the least quantified phenolic compounds and were present in traces only. To assess the effect of solvents and their concentration level, means of each phenolic compound quantified were compared statistically using the LSD test. There were significant variations observed in phenolic acids and flavonoids content with respect to solvent concentrations. Gallic acid content of 50% and 80% methanolic extracts were nonsignificant to each other, but were significantly different to other concentration levels. Ferulic acid and hesperidin at all concentration levels as well as solvent types differed significantly, whereas quercetin compound 100% methanolic and ethanolic extracts differed nonsignificantly but were significantly different from other concentration levels. Overall, 50% and 80% methanolic and ethanolic extracts lead to more phenolic compounds quantified as compared to absolute concentration levels. The antioxidant activity of mango peel extracts might be attributable to the phenolic acids and flavonoids contribution. Earlier, Chun et al [67] reported that flavonoids were responsible for the antioxidant activities of plants. Hesperidin and naringenin are the major flavanones present in orange, with the former being higher in concentration compared with the latter [42]. During a study on orange peel phenolic compounds, Khan et al [18] quantified flavanones hesperidin and naringin in orange peel extracts through HPLC and reported them as 205.20 mg/100 g and 70.30 mg/100 g fresh weight, respectively. Peels of *Magnifera indica*, *C. sinensis*, *Malus sylvestris*, and *Psidium guajava* were quantified for phenolic compounds through HPLC and compared by Zulkifli et al [56]. It was noted that all fruit peels had significantly higher concentrations of phenolic acids, especially gallic and chlorogenic acid along with flavonoids myricetin, quercetin, and kaempferol. They concluded that peels of *M. indica* and *C. sinensis* had the highest phenolic compounds and antioxidant activity.

3.5. Antimicrobial activity

Ethanolic extracts of kinnow mandarin peels were assessed for their antimicrobial activity against three foodborne bacteria (Table 3). Statistical analysis reveals that peel extracts exhibited significantly different antimicrobial potential against bacterial strains. As evident from the table, the bacterial growth inhibition activity was increased with higher concentrations of kinnow peel extracts, which implies that microbial growth inhibition is dose dependent. Kinnow mandarin peel extracts at a concentration level of 250 $\mu\text{g/disk}$ exhibited no antimicrobial activity, whereas at 500 $\mu\text{g/disk}$ concentration, slight inhibitory activity (8.67 ± 0.33) was observed against *S. aureus*. However, in the case of a kinnow peel extract at a concentration level of 1000 $\mu\text{g/disk}$, maximum inhibition zone (16.00 ± 0.58 mm) was recorded against *S. aureus* whereas minimum inhibition zone (9.00 ± 1.16 mm) was noted against *S. typhimurium*. The LSD test results reveal that there were significant differences between extract concentration against three bacterial strains. It

was observed that Gram-positive strains (*S. aureus* and *B. cereus*) were more sensitive to kinnow peel extracts as compared to Gram-negative strain (*S. typhimurium*). The variation in sensitivity among bacterial strains is ascribed to cell wall structure differences of strains. The cell wall of Gram-negative bacteria are bestowed with outer membrane as well as periplasmic space, which hinders the penetration of antimicrobial substances, thus providing more resistance to Gram-negative bacteria [68,69].

The antimicrobial activity of plant extracts may be attributed to the presence of polyphenols in extracts as high antimicrobial activity is exhibited by plant extracts with elevated polyphenol content [70]. The effects of plant extracts as antimicrobial agents depend on the polyphenol type such as phenolic acids, flavonoids, and tannins. Flavonols such as quercetin are considered potent antimicrobial agents [71]. Antimicrobial activity is substantially influenced by the position and number of hydroxyl groups because these groups may interact with the bacterial cell membrane to disrupt its structure, which leads to cellular components leakage [72]. Results are in accordance with the findings of Mathur et al [65], who reported that kinnow peel ethanolic extracts possessed maximum antimicrobial activity against *S. aureus* than other tested microorganisms. Similarly, the higher antimicrobial activity of orange, lemon, and banana peels against *S. aureus* compared with other studied bacterial, yeast, and fungal strains was observed by El Zawawy [73].

4. Conclusion

Kinnow mandarin peels are a rich source of phenolic compounds with strong antioxidant activity. UAE, which led to higher polyphenol extraction, is a more efficient technique than maceration. Absolute solvents could not ensure fair extraction of polyphenols than aqueous solvents as well as lower antioxidant activity in comparison with absolute solvents. Although methanol and ethanol are efficient solvents for extraction of polyphenols, ethanol categorized under GRAS is preferred because of its application in the food system. Strong correlations between total polyphenols and antioxidant activity were observed. Eleven phenolic compounds, including five phenolic acids and six flavonoids, were identified and quantified by HPLC. Ferulic acid and hesperidin were the most abundant whereas caffeic acid was the least quantified phenolic compounds in kinnow peel extracts. As regards the antimicrobial activity of kinnow mandarin peels against three foodborne bacterial strains, maximum inhibition zone was recorded against *S. aureus* at a concentration level of 1000 $\mu\text{g/disk}$, whereas minimum inhibition zone was noted against *S. typhimurium*. It was concluded that kinnow mandarin peels is a potential source of phenolic compounds with antioxidant and antimicrobial properties and can be utilized as an ingredient for the preparation of functional foods.

Conflicts of interest

The authors have no conflicts of interest.

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