Orange Peel: Low Cost Agro-waste for the Extraction of Polyphenols by Statistical Approach and Biological Activities

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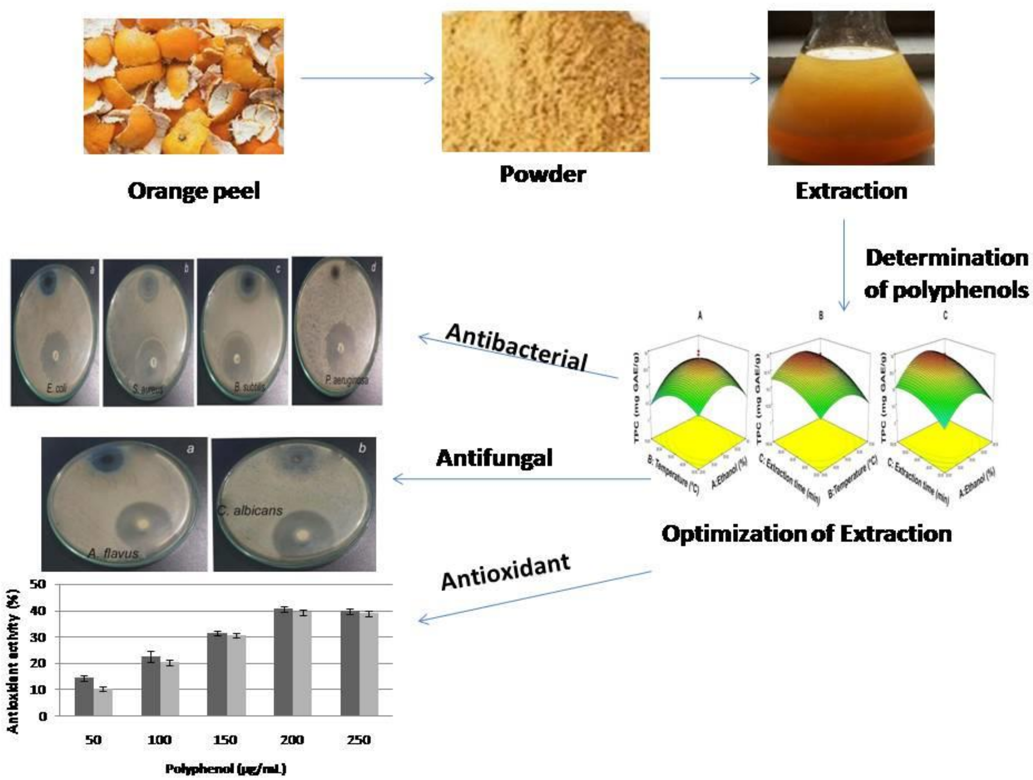
GRAPHICAL ABSTRACT



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S. Charunivedha,[a] Reem M. Aljowaie,[b] Mohamed S Elshikh,[b] T. Renisheya Joy Jeba Malar [a,*]

Food waste is an excellent source of various bioactive secondary metabolites. In addition, food waste is a global issue, and more research is being focused on the conversion of value-adding products from food waste, especially fruits. In this study, bioactive polyphenols were extracted from orange peels. The parameters that affect the extraction of polyphenols from orange peels were optimized using response surface methodology. Ethanol was used to extract bioactive polyphenols, and the extraction time (min), extraction temperature, and ethanol concentration (%) were analyzed. Orange peels are an excellent source of polyphenols, and the yield was increased under optimized extraction conditions ($p<0.05$). The polyphenol content increased twofold (38.4 mg GAE/g) under the optimized extraction conditions (53% ethanol, 51 degrees centigrade and 96 min extraction time). Crude polyphenols exhibited significant antibacterial activity against *Bacillus subtilis* (19±1 mm zone of inhibition), followed by *Staphylococcus aureus* (17±2 mm zone of inhibition) ($p<0.05$). In addition, orange peel extract exhibited antifungal activity against *A. flavus* and *C. albicans*. The ethanol extract exhibited DPPH activity (1.61±0.03 mg GAE/100 g) and ABTS reducing power (1.48±0.01 mg GAE/100 g). The polyphenol compounds exhibited antibiofilm activity against biofilm-producing *Bacillus subtilis* and increased in a dose-dependent manner ($p<0.05$).

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Keywords: Orange peel; Ethanol extraction; Polyphenols; Optimization; Antibacterial; Antibiofilm; Antioxidant

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INTRODUCTION

Food waste is a major concern throughout the world, and >1.3 billion tons of food waste are generated every year. From an environmental and economic point of view, food waste management is a foremost task. Sustainable food waste management is mandatory; moreover, it is a major challenge in developed and developing countries. Fruits are highly popular across the globe and are commonly consumed (Rampersaud and Valim 2017). The worldwide production of citrus fruits, including grapefruit, oranges, tangerines, lemon, lime, *etc*., was estimated to be approximately 158 million tons in 2019 (Ademosun 2022). Among these citrus fruits, the orange crop is a major crop and accounts for approximately 50% of the total citrus production. The pulp, seeds and peels account for 50% of the total fruit weight. Approximately 8 to 20 million tons of orange juice are manufactured annually, of which 60 to 65% (w/w) consists of orange peels (Mohsin *et al*. 2022). Orange peels are rich in polyphenols, and the extraction of polyphenol compounds from orange peels is a topic of great interest (M'hiri *et al*. 2017).

Phenolic compounds are among the major bioactive secondary metabolites and are well known for their antimicrobial, antioxidant, anti-inflammatory, anticarcinogenic, and anti-inflammatory activities (Chacar *et al*. 2017; Ahmad *et al*. 2019). In the food industry, phenolic compounds can inhibit the growth of fungi and mycotoxin production in several stored grains. Phenolic compounds are the major class of bioactive secondary metabolites predominant in several plant-based food wastes. Polyphenols have various subclasses, and these chemicals have chemopreventive properties. Polyphenols were extracted from fruit sources *via* various methods. Moreover, a green extraction method is preferable for industrial processing, and it comprises three major steps of process optimization, including the suitability of the raw materials, the energy requirement, and the solvent consumption (Ameer *et al*. 2017; Maksoud *et al*. 2021). Orange peel is a suitable raw material for the extraction of polyphenols. Extraction methods such as microwave- and ultrasound-assisted extraction require additional costs and energy for industrial-scale production (Guimarães *et al*. 2010; Sharma *et al*. 2015).Organic solvents with different polarities are widely used for the extraction of polyphenols. They exhibit several bioactive properties, including immunomodulatory, anti-inflammatory, antiaging, cardioprotective, antibacterial, antiviral and antifungal properties (Londoño-Londoño *et al*. 2010; Peña-Portillo *et al*. 2024). The objective of this study is to optimize the process parameters that affect the extraction of polyphenols from orange peels. Ethanol extraction of total phenolic compounds was performed on orange peels. The extraction time, temperature, and ethanol volume were optimized, and the total polyphenol yield was determined. Antioxidant activity was determined using FRAP (ferric reducing antioxidant power), DPPH (2,2-diphenyl-1-picrylhydrazyl), and ABTS (2,20-azino-bis-3-ethylbenzo-thiazoline-6-sulphonic acid) assays; antimicrobial and antibiofilm activities were also performed to analyze the application ethanol on polyphenols extraction.

EXPERIMENTAL

Chemicals

Gallic acid, glucose, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, and sodium carbonate were purchased from Himedia, India. The ethanol used in this study was Analytical grade with 99.9% purity.

Fruit Peel

Orange fruit (*Citrus sinensis*) was collected from the vegetable market at Kanniyakumari, Tamilnadu, India. Orange peels were cut manually at 1×1 cm, and the moisture content was removed after drying in a hot air oven at 110 °C until a constant weight was reached. The dried peels were finely ground with a blender and stored at room temperature until further use.

Extraction of Polyphenols

To extract total phenolic compounds, 10 g of orange peel powder was placed in an Erlenmeyer flask containing 500 mL of 90% ethanol. The extraction was performed for 1 h by stirring at 250 rpm at 50 °C. The mixture was further centrifuged at 5000×g for 10 min, and the supernatant was stored at −20 °C until further use.

Determination of Total Phenolic Content

The total phenolic content of the sample was analyzed using the Folin–Ciocalteu method, as described previously (Maroun *et al*. 2017). The sample (200 µL) was mixed with 1 mL of Folin–Ciocalteu reagent and 2.5 mL of sodium carbonate solution (w/w, 7.5%). The mixtures were incubated at 30±1 °C for 30 min, and color development was observed at 765 nm. To the blank, double distilled water (200 µL) was added instead of the peel extract. The total phenolic content of the extract was calculated using a gallic acid standard curve (0 to 100 mg/L). The analysis was performed in triplicate, and the results are expressed as mg gallic acid equivalent/g orange peel (mg GAE/g DW).

Determination of Total Flavonoid Content

The flavonoid content of the sample was evaluated as described previously using the $AlCl_3$ method (Sillero *et al*. 2020). Two millilitres of 2% (w/v) $AlCl_3$ was mixed with the peel extract. The blank was double distilled and mixed with $AlCl_3$. The mixture was incubated for 10 min, and the absorbance was read at 430 nm against a reagent blank. Rutin trihydrate (0 to 100 mg/L) was prepared in double distilled water and used as a standard. The results are expressed as mg rutin equivalent/g dry weight of orange peel (mg RE/g DW).

Extraction of Total Phenolic Compounds by the One-Variable-at-a-time Approach

The phenolic content of the sample was extracted using three variables. To determine the effect of extraction time, total polyphenolic compounds were extracted with 90% ethanol at 15, 30, 60, 90, and 120 min by stirring at 250 rpm. To optimize the temperature, extraction was performed at various temperatures (30, 40, 50, 60, and 70 °C) for 90 min at 250 rpm. To optimize the effect of solvent (ethanol) on polyphenol extraction, ethanol was added at various concentrations (20 to 100%, v/v). After extraction, the total phenolic compound content of the extract was determined.

Optimization of the Extraction of Total Phenolic Compounds

The phenolic content of the sample was extracted using central composite design and response surface methodology. The independent variables were extraction time (A), temperature (B), and ethanol concentration (%) (C). The variables and their levels were selected on the basis of earlier reports of polyphenol extraction via ethanol from pomegranate peels(Feng *et al*. 2022), and vegetables and fruits (Gu *et al*. 2019). The central composite design consists of 20 experiments with six central points. The variables used were 0 to 100% (v/v) ethanol, temperature (30 to 70 °C), and extraction time (0 to 120 min) (Table 1). The experiment was performed based on the designed matrix. Then, the total phenolic compound content of the extract was determined. This experiment was designed using Design Expert software, and a p value <0.05 was considered to indicate statistical significance. The predicted optimum response was used to validate the designed model. The mean value of the experimental result was compared with the predicted response, and the model was validated.

Table 1. Low and High Levels of the Selected Independent Factors

Factor	Name	Units	Low Actual	High Actual	Mean
A	Ethanol	%	20	80	50
B	Temperature	°C	30	70	50
C	Extraction time	min	20	100	60

Determination of Antimicrobial Activity

The antimicrobial activity of orange peel extract was determined against bacteria (*Escherichia coli* MTCC443, *Staphylococcus aureus* MTCC3160, *Bacillus subtilis* MTCC5981, and *Pseudomonas aeruginosa* MTCC2453) and fungi (*Aspergillus flavus* MTCC2206 and *Candida albicans* MTCC). Antimicrobial activity was determined against these microorganisms using the disk diffusion method as described previously. Briefly, bacteria (0.5 McFarland solution) were spread on Mueller Hinton agar (MHA) media. Then, the peeled extract disks soaked with 25 µL of extract were gently placed on the MHA surface. Chloramphenicol (10 µg) was used as the positive control. The culture plates were kept at 4 °C for 30 min and further incubated at 37 °C for 24 h. The zone of inhibition was determined after 24 h of incubation in an incubator. To determine antifungal activity, the selected fungal strains were spread on potato dextrose agar medium and incubated on culture plates for 72 h at 28 °C. The zone of inhibition was determined after 72 h of incubation in an incubator.

Determination of Antioxidant Activity

The total antioxidant activity of the orange peel extract was analyzed as described previously by the phosphomolybdenum method. Approximately 0.5 mL of aqueous orange peel extract or standard was mixed with 3 mL of the working reagent. This working reagent was composed of sulfuric acid (0.6 mol/L), sodium phosphate (28 mmol/L), and ammonium molybdate (4 mmol/L). The mixture was incubated for 60 min at 95 °C and cooled. The absorbance of the sample was read at 695 nm against a reagent blank. To the blank, 0.5 mL of double distilled water was added in place of the sample. Ascorbic acid was prepared at 10 to 100 mg/L, and the results are expressed as mg ascorbic acid equivalents/g dry weight of orange peels (mg AA/g DW).

DPPH Radical Scavenging Activity

Approximately 0.25 mL of peel extract was mixed with 2 mL of 0.04 mg/mL DPPH solution prepared in ethanol. The mixture was incubated for 30 min, and the absorbance was read at 517 nm against a reagent blank. For the blank, only 96% ethanol was used in place of the peel extract. Trolox was prepared at various concentrations (10 to 50 mg/L), and the DPPH radical scavenging activity was expressed as mg ascorbic acid equivalents/g of dry orange peel (mg AAE/g DW) (Miccio *et al*. 2020).

ABTS Radical Scavenging Activity

ABTS radical cations were prepared by mixing 7 mM ABTS solution with potassium persulfate (2.45 mM) at a 1:0.5 ratio. Then, the mixture was incubated at room temperature in the dark for 12 h. The solution was diluted with 70% ethanol prior to analysis. To prepare the standard curve, Trolox (100 µL), was added at various concentrations, and 0.4 mL of diluted reagent was added. The microtiter plate was incubated for 30 min in the dark at room temperature (28±1 °C). The sample was read at 734 nm against a reagent blank. The ABTS radical scavenging activity was expressed as μM TE Trolox equivalent (TE)/dry weight of orange peel (Re *et al*. 1999).

Biofilm Inhibition Analysis

The biofilm-preventing activity of orange peel extract was determined. Briefly, the 18 h bacterial culture (*B. subtilis*) was seeded into microtiter plates. The extract was added at various concentrations (10 to 100 μg/mL) in triplicate and incubated for 24 h. The optical density of the sample was measured at 600 nm. Then, the culture was washed with double distilled water, and the adhered biofilm was stained with crystal violet (0.1%, w/v). Then, the wells were rinsed with water and solubilized with acetic acid (30%, v/v), and the absorbance of the sample was read at 570 nm.

Statistical Analysis

One-way analysis of variance was used to determine the significance level. A p value<0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Effect of Extraction Time on Polyphenol Yield

According to Fig. 1, varying treatment times (min) affected the polyphenol yield, and the total polyphenol yield ranged from 2.1±0.1 to 19.2±0.72 mg GAE/g. Ethanol (>50%) and maximum extraction time were required for the extraction of almost all polyphenols from the fruit peel. The optimum extraction time was 90 min (Fig. 1). An increase in the extraction time positively affected the polyphenol yield. The results indicated that increasing the extraction time could increase the solubility of polyphenols from the fruit peel powder up to 60 min.

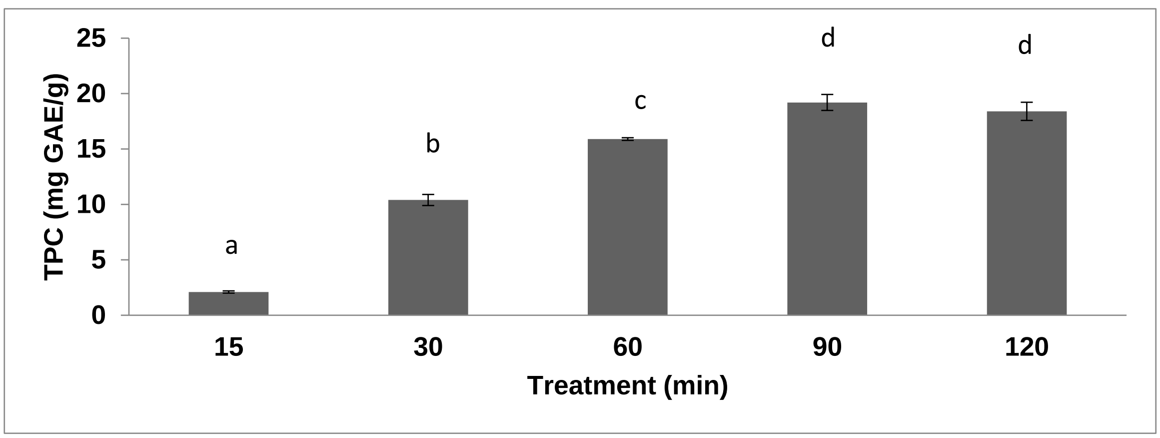

Fig. 1. Effect of extraction time on the total phenolic content of the orange peels (n=3). The values are presented as the means ± standard deviations. Mean values marked by different lower-case letters are significantly different (p<0.05).

Several researchers reported a decrease in phenolic compound yield as the extraction time increased (Tchabo *et al*. 2018; Montenegro-Landívar *et al*. 2021). Le *et al*. (2019) reported the effect of maceration time on total phenol extraction. The total phenolic compound content increased from 5.4 to 12.8 mg GAE/g of dry extract after 60 min of extraction. However, from 60 to 150 min, the total phenolic compound content remained unchanged. An equilibrium was set between the extraction solvent and the solute and was explained by Fick's second law. Once equilibrium was maintained, there was no further increase in polyphenol yield. An increased extraction time may lead to epimerization, oxidation, and degradation of plant secondary metabolites (Esparza *et al*. 2020).

Effect of Extraction Temperature on Polyphenol Yield

During extraction, the heating process increased the extraction of phytochemical components. In this study, 50 °C was found to beadequate (Fig. 2). The variation in the total polyphenol content revealed that the extraction conditions for the polyphenols were optimized. At higher incubation temperatures, the mass diffusivity increases, which improves the solute diffusivity into the solvent. A higher extraction temperature increases the mass transport of phenolic compounds and reduces the surface tension and solvent viscosity, thereby improving the phenolic compound yield (Juntachote *et al*. 2006). The heating process may, in turn, weaken the phenol-polysaccharide interactions, soften plant tissues, and other phenol interactions and promote the leaching of phytochemical compounds (Mokrani and Madani 2016). The solubility depends on the melting point of the solute, the entropy of fusion, and the activity coefficient of the solvent-solute mixture. A low melting point of the solvent and a high incubation temperature led to increased solubility. Moreover, high extraction temperatures significantly affect the stability of bioactive phytochemical compounds such as anthocyanins (Mazza and Miniati 1993). An increase in temperature from 55 °C to 75 °C affects the structure of the membrane and reduces the extract yield. In this study, longer incubation times affected the product yield; hence, the extraction temperature should be optimized (CheSulaiman *et al*. 2017).

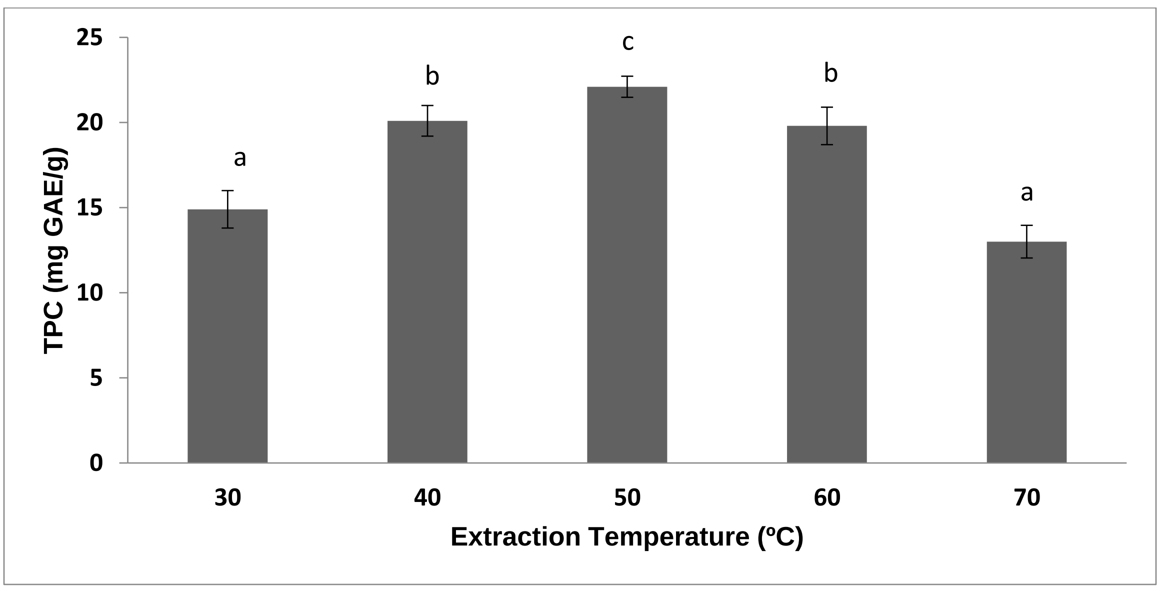

Fig. 2. Effect of extraction temperature on the total phenolic content of orange peels (n=3). The values are presented as the means ± standard deviations. Mean values marked by different lowercase letters are significantly different (p<0.05).

Effect of Ethanol on Polyphenol Compounds

The total polyphenol yield ranged from 4.8±0.2 to 25.4±0.9 mg GAE/g. The total polyphenol content reached a maximum at 60% ethanol and decreased at higher concentrations of ethanol. The present findings revealed that ethanol extraction at 60%was improved polyphenol compounds yield from orange peels (2 to 25.4±0.9 mg GAE/g) (Fig.

3) and lower concentrations of solvents increased the solubility of the phytochemicals. The polarity of the solvent should correspond with the phytochemical components of the extract (Truong *et al*. 2019).Chew *et al*. (2011) reported the effect of ethanol concentration on phenolic compound extraction from *Centella asiatica.* The amount of total phenolic compounds increased to 40% v/v ethanol. Elboughdiri (2018) extracted total phenolic compounds from olive leaves using ethanol at various concentrations (50 to 80% (v/v). In their study, the concentration of ethanol did not significantly influence the yield of phenolic compounds. Chaudhry *et al.* (2022) extracted polyphenols from banana peels at two different concentrations (50% and 75%), and 50% ethanol resulted in the maximum polyphenol yield (13.48%). Kahlaoui *et al*. (2019) extracted polyphenol compounds from almond hull powder *via* an ethanol/water mixture (70/30, v/v) for 6 h under agitation at 60 rpm. In a previous study, Feng (2022) optimized phytochemical extraction from waste orange peels via (50 to 100%) solvents, and ultrasonic assisted (40 kHz) extraction improved phytochemical yield.

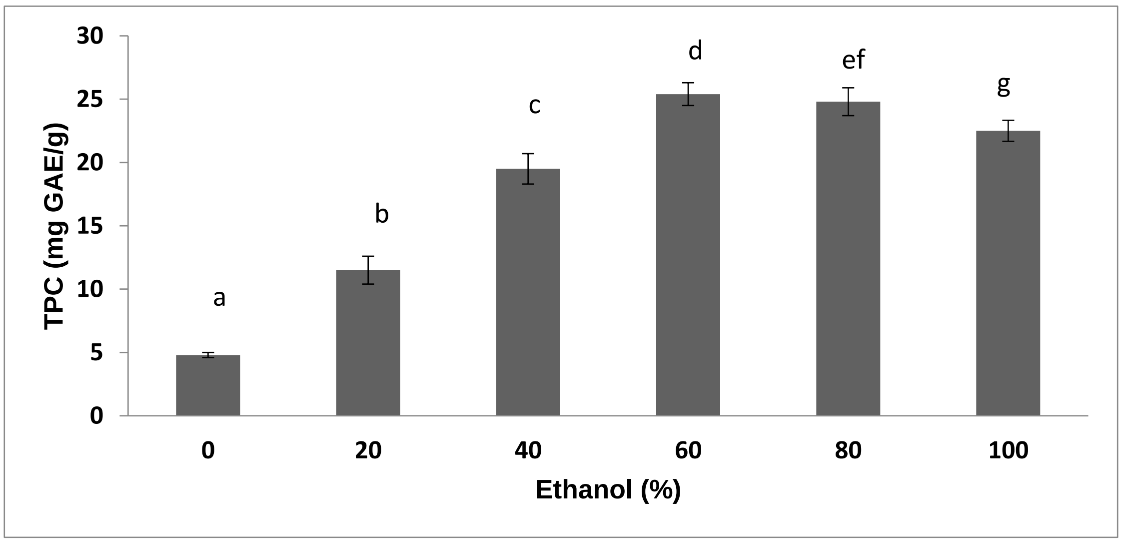

Fig. 3. Effect of ethanol concentration on the total phenolic content of orange peels (n=3). The values are presented as the means ± standard deviations. Mean values marked by different lower-case letters are significantly different (p<0.05).

Optimization of Polyphenol Extraction by Polynomial Modeling

The use of food waste to isolate polyphenol compounds is increasing and is one of the key research topics. Total polyphenols can be found in significant amounts in orange peels and can be valuable for the food processing industry. To improve polyphenol extraction, three prominent parameters were selected. These parameters included the **e**xtraction time, extraction temperature, and solvent concentration. To determine the effect of the selected experimental procedure and to improve polyphenol extraction, response surface methodology was used. A central composite design with three variables and five levels was used to analyze the effect of critical extraction variables, including ethanol concentration, extraction temperature, and extraction time. The analyzed response (polyphenol yield) was calculated, and the results are presented in Table 2. The amount of polyphenols ranged from 0.15 to 37.9 mg GAE/g.

The optimization of extraction parameters is one of the important steps, since it can minimize the application of energy, time, solvents, *etc*., and it can improve the extraction potential. Therefore, it is very important to screen factors to optimize the extraction procedure to improve phytochemical components (Athanasiadis *et al*. 2022). The selection of a suitable solvent is important because the extraction of polyphenols is based on the chemical properties of the solvent. Based on the polarity of the solvent, the extraction capacity of the solvent varied. Hence, the identification of suitable solvents at optimum concentrations is required to improve the product yield and production cost (Athanasiadis *et al*. 2022). Polyphenols exhibit medium polarity, and water is not suitable for the extraction of polyphenols from orange peels; hence, organic solvents have been frequently used to improve extraction performance (Tzima *et al*. 2015; Vázquez *et al*. 2020).

Table 2. Polyphenol Content of Orange Peels Extracted by Response Surface Methodology

Run	A: Ethanol(%)	B: Temperature (°C)	C: Extraction (min)	TPC (mg GAE/g)
1	20	30	100	14.9
2	20	30	20	1.9
3	50	50	60	30.4
4	20	70	100	6.8
5	50	16.3	60	14.9
6	50	50	-7.27	7.1
7	80	70	20	4.8
8	50	50	60	28.4
9	50	50	127.2	29.5
10	-0.45	50	60	0.15
11	50	83.6	60	1.6
12	80	30	100	8.6
13	50	50	60	35.9
14	50	50	60	37.9
15	50	50	60	30.5
16	80	70	100	31.7
17	80	30	20	6.3
18	50	50	60	30.3
19	20	70	20	3.2
20	100.4	50	60	0.48

TPC: Total phenolic content; GAE: gallic acid equivalent

Table 3. Analysis of Variance for the Extraction of Polyphenols from Orange Peels

Source	Sum of Squares	df	Mean Square	F-Value	p value Prob> F
Model	3206.806	9	356.3118	9.510369	0.0008
A-Ethanol	46.33377	1	46.33377	1.236701	0.2921
B-Temperature	4.193665	1	4.193665	0.111934	0.7449
C-Extraction time	510.1908	1	510.1908	13.61758	0.0042
AB	100.82	1	100.82	2.691001	0.1320
AC	19.845	1	19.845	0.529686	0.4834
BC	28.88	1	28.88	0.77084	0.4006
A^2	1685.236	1	1685.236	44.98088	< 0.0001
B^2	924.2533	1	924.2533	24.66938	0.0006
C^2	286.0414	1	286.0414	7.634773	0.0200
Residual	374.6561	10	37.46561		
Lack of Fit	304.3028	5	60.86056	4.32535	0.0669
Pure Error	70.35333	5	14.07067		
Cor Total	3581.462	19			

In this study, higher percentages of ethanol improved polyphenol yielded more than lower concentrations of polyphenols. In addition, other green technologies have also been used previously for the extraction of phytochemical compounds from food sources (Chatzimitakos *et al*. 2023). Table 3 shows the statistical analysis results based on the second-order polynomial equations and coefficient estimates. The selected polynomial model was best fitted, and the lack of fit was insignificant. The p-value of the model was statistically significant (p=0.0008). Among the factors, extraction time (p-value=0.0042) was significant.

The increase in ethanol volume in the mixture and extraction time influenced the degree of phytochemical extraction. The coefficient estimates for these two variables were positive, which indicated that the selected variable ranges were appropriate (Arasu *et al*. 2019; Benito-Román *et al*. 2020). However, higher temperatures affected the product yield (polyphenols), and the coefficient estimate was negative. The results were analyzed by multiple regression fitting and quadratic polynomial regression model equations based on the coded values. The equations were used to analyze the relationships between the three independent variables and the value of the response. The equation in terms of coded variables can be applied to make predictions about the response (Y) for given levels of each variable. The coded equation is applied for determining the relative impact of the variables by comparing the factor coefficients. The final equation in terms of coded factors was as follows:

$$TPC = +32.16+1.84A-0.55B+6.11C+3.55\,AB+1.58\,AC+ 1.90BC-10.81A^{2}-8.01\,B^{2}-4.46\,C^{2}$$

The mathematical second-order polynomial model equations of the selected variables were analyzed, and the R^2 value was greater than 0.95. The lack of fit value was insignificant. Thus, the present findings revealed that the models fit the results well and that the selected process variables accounted for at least 95% of the variability in polyphenol yield. Therefore, only 5% of the variability may be attributed to other variables. The 3D-response surface graph derived from the models shows the visual effect of the selected variables on the polyphenol yield (Alberti *et al*. 2014). Compared with temperature, the concentration of ethanol significantly increased the total phenolic compound yield. Similarly, extraction time significantly increased the total phenolic compound yield compared with the ethanol volume (Chaudhry *et al*. 2022; Feng *et al*. 2022). Compared with the extraction time, the incubation temperature marginally increased the total phenolic compound concentration (Fig. 4).

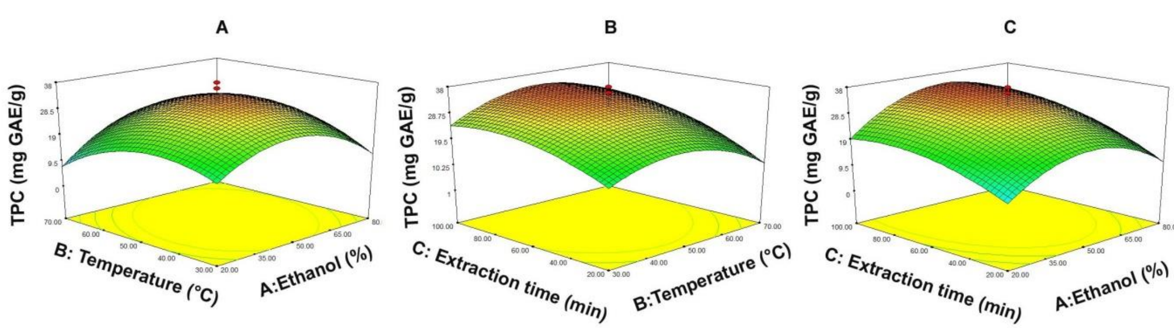

Fig. 4. Three-dimensional response surface graph showing the response (total polyphenol yield) as a function of process factors. (A) Extraction with ethanol and temperature, (B) ethanol and extraction time, (C) temperature and extraction time

The predicted optimum response was optimum at 53% ethanol, 51 degrees centigrade and 96 min of incubation, and the corresponding total polyphenol yield was 39.1 mg GAE/g. An experiment was conducted to analyze the optimum value based on the predicted response. Under optimum conditions, the experimental value was 38.4 mg GAE/g, which was close to the predicted value (39.1 mg GAE/g), which validated the designed central composite model.

Antibacterial Activity of Orange Peels

Fruit waste is extensively used as a major source for the extraction of bioactive secondary metabolites. Crude plant extracts are used to treat various bacterial and fungal pathogens (Tripathi *et al.*2024). However, the exact mechanism of action of antibacterial secondary metabolites is still unknown. Polyphenols interact with bacterial enzymes at the cellular level, affect the permeability of the cell membrane and affect the integrity of the bacterial cell wall. Figure 5 depicts the effect of polyphenol compounds on the bacterial strains. The results showed that under the selected extraction conditions, the orange peel extracts had antimicrobial effects on selected bacteria and fungi. The antimicrobial activity varied among the microorganisms. The inhibition activity of the ethanol extract of the orange peels was greatest against *B. subtilis* (19±1 mm zone of inhibition), followed by *S. aureus* (17±2 mm zone of inhibition) (Figs. 5 and 6).

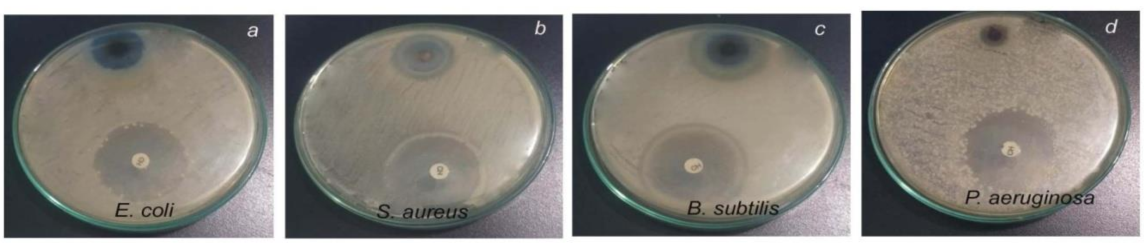

Fig. 5. Antibacterial activity of orange peel extract against *E. coli* (a), *S. aureus* (b), *B. subtilis* (c), and *P. aeruginosa*. The bacterial culture was swabbed on Mueller Hinton agar medium, and the extract was loaded on a disk. The zone of inhibition was measured after 24 h of incubation.

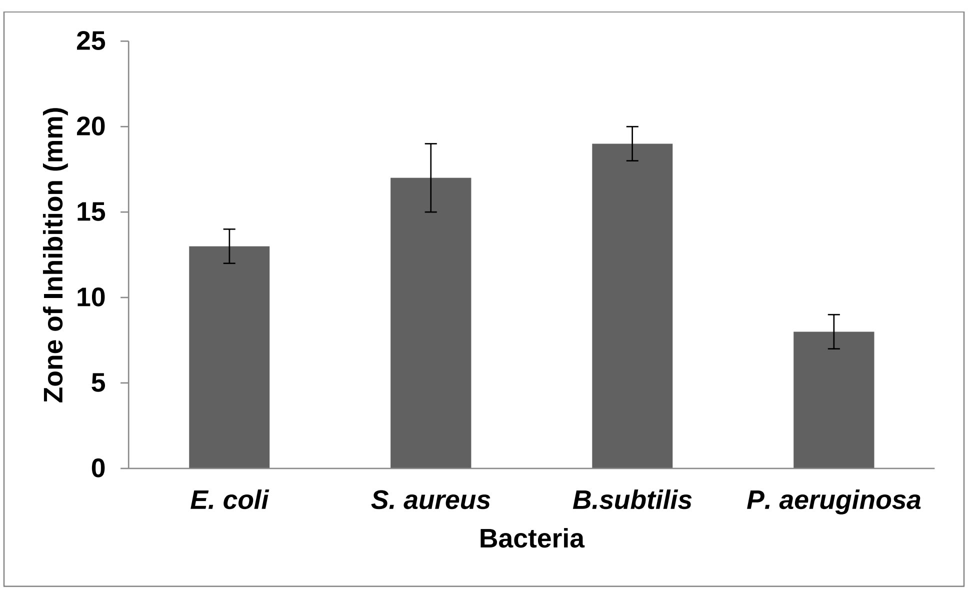

Fig. 6. Antibacterial activity of orange peel extract. The zone of inhibition is expressed as the mm zone of inhibition, and the error bars represent the standard deviation.

Shehata *et al*. (2021) reported the antibacterial activity of citrus peel extract against *Bacillus cereus* ATCC 49064, and a maximum zone of inhibition was observed, which was similar to the results of this study. The polyphenol content of the extract in the citrus fruit peels exhibited antimicrobial activity. In this study, the fruit peel extract showed the least activity against *Pseudomonas aeruginosa,* and the zone of inhibition was 8±1 mm.

Antifungal Activity of Orange Peel Extract

The orange peel extract exhibited antifungal activity against *A. flavus* and *C. albicans*. The zone of inhibition was 13±1 mm for *A. flavus* and 15±0 mm for *C. albicans*(Fig. 7). Plant secondary metabolites include bioactive compounds such as polyphenols and flavonoids, and these compounds directly affect the growth of fungi. Fruit peel extract has antifungal potential because the available phytochemical compounds may bind to cell membranes *via* hydrogen bonding and hydrophobic interactions, thus affecting membrane permeability (Gonelimali *et al*. 2018). In addition, flavonoids contain hydroxylated phenolic substances that are responsible for antimicrobial activities. Plants produce flavonoids in response to bacterial or fungal infections, and flavonoids are highly effective against a broad range of microorganisms. Flavonoids interact with microbial cellular proteins and form complexes that affect cellular functions. Flavonoids use nonspecific mechanisms and act on the respiratory chain or on the bilayer of the microbial cell membrane (Yuan *et al*. 2021).

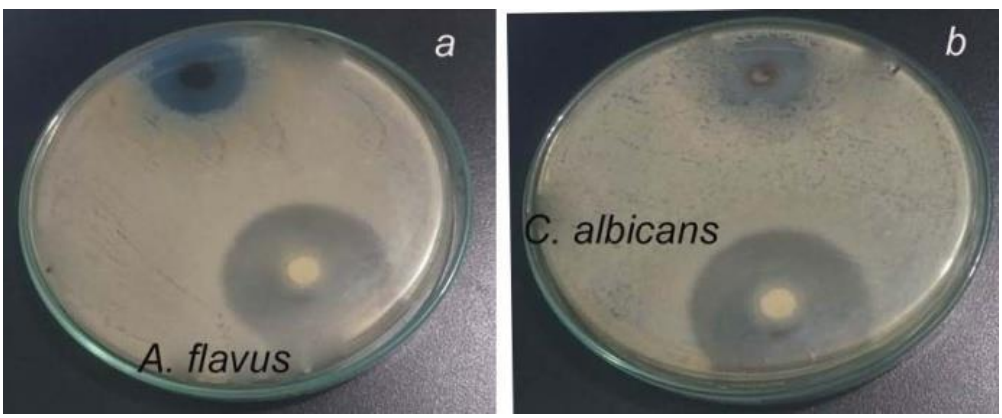

Fig. 7. Antifungal activity of orange peel extracts against *Aspergillus niger* (a) and *Candida albicans*

Antioxidant Activity

The total antioxidant activity, DPPH activity, and ABTS reducing power of the solution were assessed. The ethanol extract exhibited significant antioxidant activity in all three selected methods of analysis. The antioxidant activity of the peel extract was 1.93±0.03 mg GAE/100 g. The DPPH activity of the orange peel extract was 1.61±0.03 mg GAE/100 g. The ABTS reducing power of the orange peel powder was 1.48±0.01 mg GAE/100 g. The antioxidant activity of the polyphenol compound was dose dependent (Fig. 8). At a concentration of 100 µg/mL, the antioxidant activity reached a maximum, and the variation was significantly different among the phytochemical concentrations. The antioxidant power of the extract varied based on the phytochemical component of the extract. The alcoholic blood orange extract contained antioxidant phytochemicals such as flavonoid glucosides, flavonoids, and limonoids. In this study, the antioxidant activity determined by the ABTS assay method was highly consistent with that determined by the

DPPH assay. In this study, two antioxidant assays were performed, and at least two antioxidant mechanisms were evaluated to determine the mode of action of the antioxidant phytochemical components (Babbar *et al*. 2011). The antioxidant activity of phenolic compounds extracted from *Citrus sinensis* L. and *Citrus aurantium* L. peels by the methods of Lagha-Benamrouche and Madani (2013) has been reported. Selahvarzi *et al*. (2022) optimized the ultrasonic-assisted extraction of phytochemical compounds from orange peels and pomegranate peels and reported their antioxidant activity.

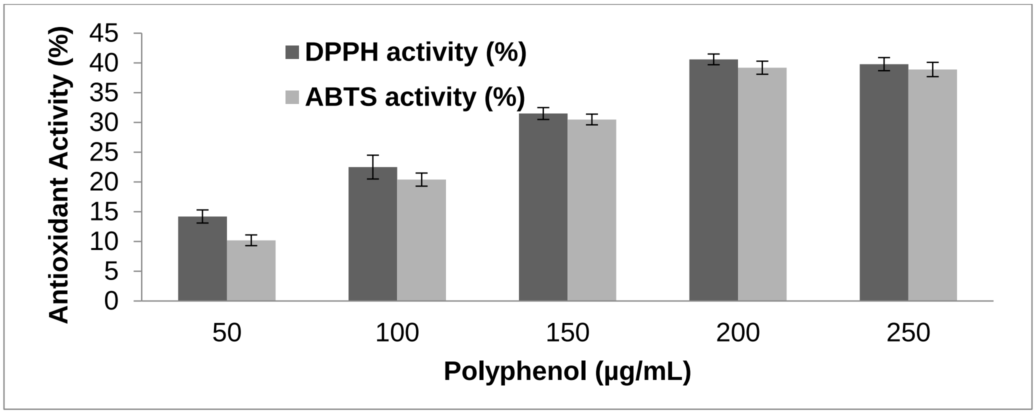

Fig. 8. Determination of the antioxidant activity of orange peels extracted from the peels by ethanol *via* DPPH and ABTS assays

Antibiofilm Activity of Polyphenol Compounds from Orange Peels

Biofilm inhibition analysis was performed against biofilm-forming *B. subtilis*. The culture was treated with various concentrations of plant extracts, and the antibiofilm activity was determined after 24 h of treatment. The polyphenol compounds exhibited antibiofilm activity, which increased in a dose-dependent manner. A schematic of the antibiofilm activity of orange peel extract is shown in Fig. 9.

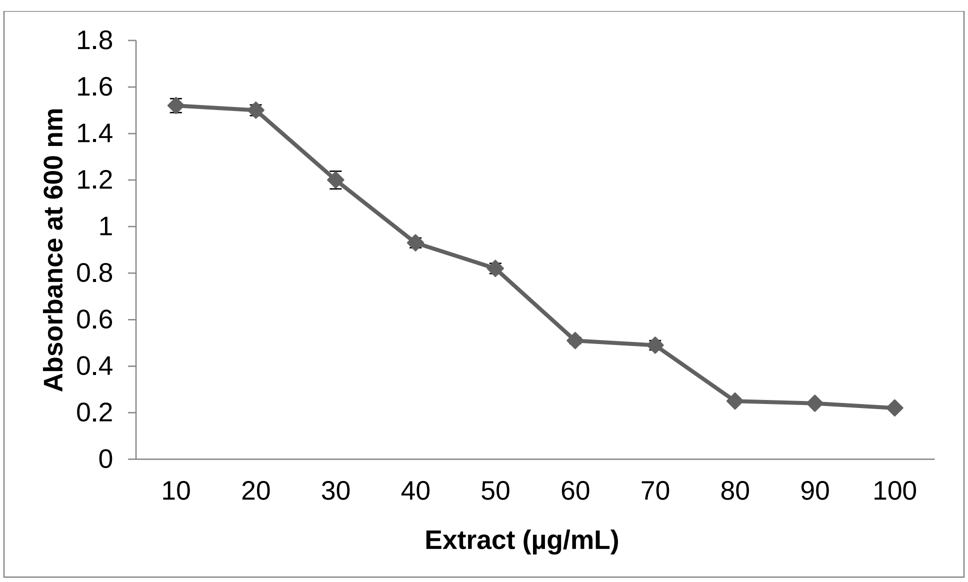

Fig. 9. Antibiofilm activity of orange peel extract at various concentrations. *Bacillus subtilis* was cultivated for 24 h in nutrient broth media supplemented with various concentrations of orange peel extract.

Antibacterial and antibiofilm compounds were reported from several plant sources. Sublethal doses of plant-derived compounds have been found to affect bacterial adhesion and biofilm formation while inducing planktonic growth of bacteria with a hypermotile phenotype (Alav *et al*. 2018; Deepika *et al*. 2018). This biofilm activity can be applied to disarm bacteria of sanitary interest without killing them or opposing selective pressure on the bacteria but increasing susceptibility or reducing virulence (Reen *et al*. 2018). Polyphenols exhibit antimicrobial activity on biofilms synthesized from *Staphylococcus epidermidis* (Ferrazzano *et al*. 2011). Villa and Cappitelli (2013) reported that bioactive secondary metabolites block quorum sensing signaling systems and affect the development of biofilms in most clinically significant bacterial pathogens. Bioactive secondary metabolites such as flavonoids in citrus peels exhibited significant activity against *Streptococcus mutans*, whereas phenolic acids showed activity against the drug-resistant *Pseudomonas aeruginosa* PA1 (Yue *et al*. 2018).

CONCLUSIONS

1. Orange peel powder was used as a cheap source for the extraction of bioactive compounds. The extraction procedure was optimized using response surface methodology. The optimized extraction protocol (53% ethanol, 51 degrees centigrade and 96 min extraction time) improved the polyphenol yield twofold.
2. The orange peel exhibited antimicrobial activity against bacterial and fungal pathogens. The polyphenol compounds exhibited antibiofilm activity against biofilm-producing *Bacillus subtilis*. The polyphenol compounds exhibited antioxidant activity.
3. More research must be performed on extracting bound phenolic compounds from agro by-products. The extraction methods for phenolic extracts require many on *in vitro* and *in vivo* analysis, and their complete mechanisms need to be explored.

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