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Research Article
Phytochemical Investigation and Antimicrobial Activities of Jordanian *Psidium guajava* Raw Fruit Peel Extract using Soxhlet and Microwave Extraction Methods

[1]Reem A. Issa, [2]Wamidh H. Talib and [1]Maha Habash

[1]Department of Pharmaceutical Chemistry and Pharmacognosy,
[2]Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Science University, Amman, Jordan

Abstract

Background: This study was undertaken to evaluate the phytochemical composition of the aqueous extract obtained from Jordanian premature *Psidium guajava* fruit peel (PGFP) and their antimicrobial effect, using two different methods for extraction. **Materials and Methods:** Two different methods for extraction were used: Microwave Integrated Soxhlet (MIS) extraction and Conventional Soxhlet (CS) extraction. The TLC analysis was used to optimize each extraction method and for the preliminary phytochemical screening. The HPLC-MS/MS was used for the identification and the quantification of the phenol compounds in the plant. The antimicrobial activity of both aqueous PGFP extracts was measured using micro-titer plate dilution method. **Results:** Four phenol compounds were identified in the MIS extract, of which ascorbic acid showed the major constituent, while six phenol compounds were identified in the CS extract, of which ellagic acid was the major constituent. Moreover, the MIS extract contained higher levels of total phenol compounds compared to the CS extract. According to the antimicrobial assay, the two extracts of PGFP showed inhibitory activity against both Gram-positive and Gram-negative bacteria. The Minimum Inhibitory Concentration (MIC) of MIS extract against *Escherichia coli, Staphylococcus aureus, Propioni bacterium acnes* and *Bacillus cereus* was twice less than the values obtained from the CS extract, except for *S. aureus*, where the two extracts were equally active. **Conclusion:** Optimization of the extraction method used is required in order to increase the content of the polar portion in the extract, which showed to have the highest antimicrobial activity. These data can be utilized in developing combined remedies composed of synthetic antibiotics with phytochemicals obtained from *P. guajava* peel extracts, which can be used for different purposes; such as functional food development and in food preservative.

Key words: Antimicrobial, *Psidium guajava*, microwave, extraction, phenol

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Corresponding Author: Reem A. Issa, Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Science University, P.O. Box 926296, 11931 Amman, Jordan Tel: +96265609999/00962-79-7510797 Fax: +96265232899

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Traditionally, the plant *P. guajava* showed various biological activities including antioxidant, hepatoprotective, anti-allergy, ant-imicrobial, ant-ispasmodic, anti-cough, anti-diabetic and anti-inflammatory activities *in vitro* and/or in animal models, indicating the immense potential of this plant in treatment of many diseases[1,2]. The composition of the metabolites in this plant varied in the literature, depending on using different extraction protocols (temperature, source of energy and solvent), also on the origin of the plant, growing conditions, drying process and sample pretreatment method[3].

Psidium guajava fruit are considered as a highly nutritious fruits, contains sugar, vitamins A and B, proteins and mineral salts, mainly iron, calcium, phosphorus and pecticpectins, polyphenol compounds, ellagic acid, guiajaverin and quercetin[4,5]. Also, higher vitamin C content in *P. guajava* fruit than in citrus have been reported (80 mg of vitamin C in 100 g of fruit)[6]. That was highly concentrated in the skin of the fruit, secondly in the flesh and little in the central pulp. Bashir and Abu-Goukh[7] found ascorbic acid and phenol compounds content decreased continuously during repining of the fruit, the unripe peel showed higher values of ascorbic acid, total protein and phenol compounds than in the pulp.

Up to our knowledge, there are no available data in the literature studying the locally harvested unripe PGFP chemistry. Therefore, MIS and CS extraction methods were used for this investigation. The MIS method has the advantages over other commonly used extraction methods of being more environment friendly with minimum addition of solvents, fast and economic technique and with minimum energy waste[8,9]. In addition, the antimicrobial actives for each extract were evaluated, using different microbial strains.

MATERIALS AND METHODS

Plant materials: Unripe fruits of *P. guajava* obtained from the local market in Amman city, capital of Jordan; which was collected from Jordan valley during the autumn season (September-November, 2013) were used. A voucher specimen of *P. guajava* was compared with that deposited in the Herbarium of the University of Jordan (code ASU August/2013; voucher No. RI 3/2013).

The raw fruit were peeled off with a knife and the thin greenish peel was cut into small pieces and deposited in freezer at 20°C in the laboratories of the Faculty of Pharmacy, Applied Science University, Jordan for later use.

Samples extraction

CS extraction: The peel pieces were mechanically crushed and 30 g were continuously extracted for ~8 h in 150 mL distilled water with total 3-4 extraction cycles. The extract was filtered and concentrated in rotatory evaporator at 35±5°C under reduced pressure, to obtain semisolid material. The concentrated extract was lyophilized and stored at 4°C until used.

MIS extraction: Extraction was performed inside a microwave oven (Sharp [M-340B], Thailand). This oven has a frequency of 2450 MHz with a maximum delivered power of 1000 W.

A convenient hole was opened on the microwave oven. The hole was enclosed with PTFE to prevent any leakage of the inner heat; the dimensions of the PTFE-coated cavity are (30×30×20 cm). A clevenger apparatus was settled in the hole and connected with sample flask. A cooling system outside of the microwave cavity condensed the distillate continuously. The temperature was controlled with the aid of a shielded thermocouple inserted directly into the sample container at 78°C inside the oven and at -1°C for the cooling system. The MIS procedure was performed at atmospheric pressure.

The peel pieces were mechanically crushed and 60 g were loaded in MIS with 50 mL distilled water. The cooling system was seated for extraction time of 20 min per cycle. This cycle was done in triplicate and then the total extracts were combined, filtered and lyophilized, then stored at 4°C until used.

Chromatographic analysis

TLC analysis: In aim to determine the optimal time required for each method of extractions, TLC analysis for PGFP extracts obtained at different time intervals, were performed as previously done by Dhiman *et al.*[10]. Qualitative chemical screening for the identification of various classes of active chemical constituents (carbohydrates, tannins, saponins, cardiac glycosides, amino acids, flavonoids, volatile oils and alkaloids) were investigated. Phytochemical analysis was done using three different chromatographic systems; ethylacetate:formic acid:glacial acetic acid:water (100:11:11:24), ethanol:water (50:50), isopropanol:ethyle acetate:water (70:10:20) were all developed on pre-coated Thin Layer Chromatography (TLC) plates (Silica gel 60F-254) with the adsorbent layer thickness of 0.25 mm (E-Merck) were used. Detection of the separated spots was done according to standard methods described by Trease and Evans[11].

HPLC-MS/MS analysis: Chromatographic separation of PGFP extracts were performed on a reversed phase thermohpersil-keystone, DBS Hypersil C8 column (150×4 mm, 5 µm) using an Agilent 1200 LC system (Agilent, Santa Clara, CA,USA) equipped with degasser (G1379B), binary pump (G1312A) along with autosampler (G1367B). The autosampler was maintained at 6°C and programmed to draw 20 µL of sample for chromatographic separation.

An isocratic mobile phase of deionized water: 0.01% TEA/methanol (40:60, v/v) was applied at a flow rate of 1.0 mL min^{-1}. The column temperature was kept at 27°C. The total analytical run time was 6.0 min for each sample. Detection was carried out on an AB Sciex (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) API-3200 Q-Trap mass spectrometer, equipped with a turboionspray interface operated in negative ion mode (ESI). Quantification was performed using Multiple Reactions Monitoring (MRM) method. Instrument parameters optimized were collision-activated dissociation gas (CAD): Medium flow, curtain (CUR) gas: 24 psi, nebulizer gas (gas1): 30 psi, heater gas (gas 2): 25 psi, ion spray voltage: -4500 V and source temperature: 550°C. Compound dependent voltage parameters were as listed in Table 1. System control and data analysis were performed by AB Sciex Analyst software (version 1.5). The expected phenol compounds were chosen base on previous studies[2,3,12-14].

All samples were re-dissolved with known sufficient amount of water and then completed with water and methanol with a final diluent composition of (1:3, water:methanol). The dilution process (volume of diluent needed) depends on the produced retention times, where there should be a match in retention times between the standards and the extracted analytes.

Determination of phenol compounds content: Quantification of the detected phenol compounds was performed using area under the peak of the isolated ions and the established one point calibration of the corresponding standard compounds. Analyte final concentrations were determined as (mg kg^{-1}) fresh weight of plant material. All samples were analyzed in triplicates. The log p-value (the logarithm of the partition coefficient between two phases) for each detected compound was calculated using Marvin View (Version 5.1.4), Chem Axon Ltd., Budapest, Hungary.

Total content of phenol compounds in each extract were calculate as the sum of concentrations of each compound. The percentage of each phenol compound in each extract was calculated as its relative content to the total sum of all phenol compounds in the extract.

Determination of anti-microbial effect

Microbial strains: The microbial strains used in this study were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC25213), *Bacillus cereus* (ATCC10987) and *Propioni bacterium acnes* (ATCC 6919). According to the method described by Talib *et al.*[15] bacterial strains (except for *P. acnes*) were stocked onto nutrient agar slant at 4°C. *Propioni acnes* were stocked onto reinforced clostridial agar at 4°C.

Antimicrobial assay: The antimicrobial activity of both aqueous PGFP extracts (CS and MIS) was measured using microtiter plate dilution method[16]. Sterile flat bottom 96-well microplates (Nunc) were used in this method. Stock extracts were initially dissolved in distilled water, sterilized by filtration using 0.2 µm filters and subsequently diluted using Muller Hinton broth (MHB) (Oxoid, UK). Positive controls of tetracycline and penicillin G (Oxoid, UK) were prepared at the following concentrations 0.04, 0.07, 0.15, 0.20, 0.25, 0.35 and 0.45 mg mL^{-1} as described by Al-Sinjilawi *et al.*[17]. Extracts were diluted to twice the desired initial test concentration (20 mg mL^{-1}) using MHB. All wells, except the first were filled with MHB (100 µL). According to the method described by Talib and Mahasneh[18] two hundred micro liters of each extract were added to the first well and serial two-fold dilutions were made down to the desired minimum concentration (0.14 mg mL^{-1}). An over-night culture of bacteria suspended in MHB (except for *P. acnes* which was suspended in reinforced Clostridial Broth, (Oxoid, UK) was adjusted to turbidity equal to 0.5 McFarland standard. The plates were inoculated with bacterial suspension (100 µL $well^{-1}$) and incubated at 37°C for 24 h. *Psidium acnes* was incubated under anaerobic conditions using an aerogens bags (Oxoid,

Table 1: Expected phenol compounds in PGFP with their MS, MS-MS and dependent voltage parameters

Analyte	MS (m/z)	MS-MS fragment (m/z)	DP	EP	CE	C×P
Ellagic acid	300.6	200.9	-64	-5	-42	-1
Gallic acid	168.8	124.9	-50	-5	-20	-2
Quercetin	300.9	150.9	-50	-5	-27	-1
p-coumaric acid	162.7	118.8	-33	-5	-20	-2
Ferulic acid	192.9	133.9	-35	-6	-23	-2
Ascorbic acid	174.9	114.9	-37	-5	-17	-2

UK). Then the turbidity was measured using micro-plate reader (Biotek) at a wavelength of 600 nm. Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of each e7x tract that causes complete inhibition (100%) of each microbial strain.

Cytotoxicity assay: The cytotoxicity of aqueous extracts against Vero (African green monkey kidney, ATCC: CCL-81) cell line was evaluated using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Promega, Madison, WI, USA) as described by Talib and Mahasneh[19]. Vero cells were grown using Minimum Essential Medium Eagle (Gibco, UK) supplemented with 10% heat inactivated fetal bovine serum (Gibco, UK)[20], 29 μg mL^{-1} L-glutamine and 40 μg mL^{-1} gentamicin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C as described by Talib and Mahasneh[18]. Exponentially growing Vero cells were washed and seeded at 15,000 cells per well in 96 well microplates (Nunc). A partial monolayer was formed after 24 h incubation, then the media was removed and 200 μL of the medium containing the plant extract were added and incubated for 48 h. At the end of the incubation period, 100 μL of the medium were aspirated and 15 μL of the MTT solution were added to the remaining medium (100 μL) in each well. Blue crystals were produced after 4 h contact with MTT solution. Crystals were dissolved by adding 100 μL of the stop solution and incubation for 1 h.

Color intensity produced by reduced MTT was assayed at 570 nm using a microplate reader (Biotek, USA). Cells incubated with culture medium were used as a negative control while, cells treated with vincristine sulfate were used as a positive control.

The IC_{50} values were calculated as the concentrations that cause 50% inhibition of proliferation on Vero cells and were reported as the average of three replicates.

RESULTS

Comparison between the MIS and CS extraction methods: Table 2 describes the differences between the two extracts obtained using different extraction methods. The percentage yield of the extraction (with respect to the starting fresh plant material) using the CS extraction method was approximately twice the yield obtained using the MIS method. Nevertheless, MIS method was more time and energy efficient.

TLC analysis: In the preliminary phytochemical study, TLC analysis for each extract type showed the presence of phenols, carbohydrates, saponins, amino acids, tannins, volatile oils, alkaloids and flavonoids in each extract with different composition (Table 2).

HPLC-MS/MS analysis: The profile of the phenol compounds in each PGFP extract was determined using HPLC-MS/MS analysis, showed six of the expected phenol compounds in the CS extract, while only four compounds were determined in the MIS extract (Table 3).

Comparison of phenol compounds content between the two extract: Table 3 showed the content of phenol compounds in each extract using the HPLC-MS/MS method. Ellagic acid was found as the major compound in the CS extract, followed by p-coumaric acid, gallic acid, ferulic acid, quercetin and ascorbic acid at low concentration. On the contrary, ascorbic acid was found as the major compound in the MIS extract at a concentration of approximately 10 times higher than its concentration in the CS extract, followed by gallic acid, were quercetin and p-coumaric acid were found at low concentrations, approximately less than half its level in the CS extract.

In addition to the qualitative and quantitative differences in the individual phenol compounds, significant differences in

Table 2: Effect of extraction methods (CS and MIS) on the physical characterizations and chemical composition of PGFP extracts

Extract characters	CS method	MIS method
Wight of fresh plant material/cycle	30 g	60 g
Extraction time	8 h	1 h
Averaged percentage of yield	4.8%	2.5%
Extraction temperature	90-100°c	78-88°c
Lyophilized extract color	Caramel brown powder	Whit to ivory powder
Phenol	+++	+++
Tannins	++	++
Saponins	+	+
Cardiac glycosides	-	-
Carbohydrates	+++	++
Flavonoids	++	+++
Alkaloids	+	+
Amino acids	++	++
Volatile oils	++	++

-: Absent, +: Slightly present, ++: Moderately present, +++: Highly present

Table 3: Yield (mg kg^{-1}) of fresh weight plant material, percentage (to the total sum of phenols) and log p-value for each detected compound in the PGFP extracts using CS and MIS extraction methods

		CS		MIS	
Metabolites	Log P#	(mg kg^{-1})*	%**	(mg kg^{-1})*	%**
Ellagic acid	2.32	12.1	30.86	ND	------
Quercetin	2.16	2	5.10	0.7	0.35
p-coumaric acid	1.83	8.3	21.17	3	1.50
Ferulic acid	1.67	7.2	18.36	ND	-------
Gallic acid	0.72	8	20.40	26.4	13.26
Ascorbic acid	-1.91	1.6	4.08	169	84.88
Total		39.2	-------	199.1	-----

#Calculated using MarvinView (Version 5.1.4), ChemAxon Ltd., Budapest, Hungary, *Yield for the metabolite determined as (mg kg^{-1}) of fresh weight of plant material, **Percentage of metabolite to the total sum of phenols in the extract, ND: Not detected

Table 4: Minimum inhibitory concentration (MIC) in mg mL^{-1} of aqueous extracts of PGFP prepared using CS and MIS extraction against various microbial strains

	Microbial strains			
Test solution	*Escherichia coli*	*Staphylococcus aureus*	*Propioni bacterium acnes*	*Bacillus cereus*
MIS extract	10	10	10	5
CS extract	20	10	20	10
Penicillin G	0.25	0.35	0.20	0.07
Tetracycline	0.15	0.15	0.15	0.07

the total sum of phenol compounds between the two PGFP extracts were observed. The MIS extract shows to contain a total of 199.1 mg kg^{-1} of phenol compounds. That is approximately 5 times higher than its level in the CS extract.

Antimicrobial activities: Investigating the antimicrobial activity of PGFP extracts involved a comparison of the two types of extracts with the commercially available antibiotics (Table 4). The reported MIC values for each extract type, clearly shows that MIS extract is effective twice as the CS extract against all the tested bacteria except for *S. aureus*, both extracts type were equally active. On the other hand, we have noted that the commercial antibiotics showed a larger inhibitory effect than the two types of PGFP extracts. This is not surprising and reinforces the position that commercially available antibiotics may be used in treatments in combination with phytochemicals, in order to increase their efficiency and decreasing the continuously emerged bacterial resistance to antibiotics.

Cytotoxicity effects: Investigating the cytotoxicity effect of PGFP extracts involved a comparison of the two types of extracts, with the commercially available vincristine sulfate (Table 5) for their ability to induce cell death in Vero cells. The reported IC_{50} values for each extract type showed that both extracts were equally safe, with similar IC_{50} values of 23 and 25 mg mL^{-1} for CS and MIS extracts, respectively. In comparison with vincristine sulfate (IC_{50} = 0.25 mg mL^{-1}), PGFP extracts were approximately 100 times less toxic than this anticancer agent which indicates the potential use of these extracts in different therapeutic purposes without toxic side effects.

Table 5: IC_{50} (mg mL^{-1}) of PGFP aqueous extracts, prepared using CS and MIS extraction against vero cells

Test solution	IC_{50} (mg mL^{-1})
MIS extract	23±1.2
CS extract	25±0.89
Vincristine sulfate	0.25± 0.03

DISCUSSION

This is the first study to compare PGFP aqueous extracts, prepared using MIS and CS extraction methods, for their antibacterial activities against the tested microbial strains (both Gram positive and Gram negative strains). Nevertheless, the MIS extract was found to be more potent in its antimicrobial effects. The cytotoxicity study showed a large safety profile for these extract contents. However, limited studies had identified the individual type of these antimicrobial compounds.

In a similar study by Biswas *et al.*[21] observed a significant inhibition of Gram positive bacteria (*B. cereus* and *S. aureus*) but not on the Gram negative bacteria (*E. coli* and *S. entertidis*) using guava leaves, when extracted using methanol or ethanol as the solvent (moderate polarity), better than n-hexane (organic solvent) and water (highly polar) for the extraction of the antibacterial compounds. These data agree with our results as the CS extract showed a significant inhibition of the tested Gram positive bacteria, with MIC

equivalent to half the concentration required for inhibiting the growth of the tested Gram negative bacteria. On the contrary, the MIS extract showed equal growth inhibitory effect on all the tested bacterial strains, except for *B. cereus*, where half the MIC was measured.

Our phytochemical analysis using HPLC-MS/MS method, revealed that these effects might be due to the abundance of ascorbic acid; in significantly high concentrations (absolute and percentage content) in the MIS extract, compared to the CS extract, in combination with the second major compound, gallic acid. A similar finding were proposed by Tajkarimi and Ibrahim[22], in their study they suggested that application of ascorbic acid, in combination with lactic acid, may have potential as food preservative to inhibit the growth of *E. coli*.

On the contrary, the shown antimicrobial activity for the CS extract is due to the high content of ellagic acid, which was suggested that its antimicrobial affectivity is determined by increasing the permeability of the tested microbial strains[23]. In addition, it was combined to the second major phenol compound p-cumaric acid, which showed to completely inhibit the growth of the tested microbe, when studied by Aziz *et al*.[24].

This study suggests that *P. guajava* possesses compounds containing antibacterial properties and that they are study in synergism. That most of these active compounds were in the MIS extract, which has the most polar fraction of the extract. These differences in extracts composition were largely determined by the method of extraction used, source of energy and the polarity of the constituents in the extracted plant material. Ascorbic acid, has the highest polarity (lowest log p-value), while ellagic acid has the lowest polarity (highest log p-value) among the other detected phenol compounds. These findings agree with our expectations as it is known that using microwave energy for extraction would increase the solubility of the more polar compounds, compared to the conventional extraction method[25].

CONCLUSION

These findings suggest that PGFP extracts can be useful in controlling *E. coli*, *S. aureus*, *P. acnes* and *B. cereus* infections and possibly can be useful in improving the activity of some cell wall synthesis inhibitor antibiotics by acting in different mechanisms. This may be explained by the possible synergism effect between different phenol compounds, rather than the effect of an individual compound in the extracts. Therefore, optimization of the extraction method is required in order to increase the content of the polar portion in the extract, which showed to have the highest antimicrobial activity.

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