Peels of Psidium guajava fruit possess antimicrobial properties

Abstract

Objective: Psidium guajava (guava) leaf extracts have been extensively studied for their antimicrobial effect. Yet, very few studies investigated the antimicrobial effect of the ripe guava fruit. This study aims at examining aqueous extracts of ripe Psidium guajava fruit, bulb, seeds and peels (harvested in autumn at Jordan River valley).

Materials and methods: Decreasing concentrations of water extracts of three parts of the fruit were tested against Gram positive and Gram negative bacteria as well as the yeast Saccharomyces cerevesea using the agar diffusion method. Clinical isolates of methicillin resistant Staphylococcus aureus (MRSA) were also included.

Results: Water extracts of Guava peels at concentrations ≥ 10% were inhibitory to coagulase positive S. aureus and MRSA strains, and at ≥1% were inhibitory to coagulase negative Staphylococci. Water extracts of peels acted synergistically with cell wall synthesis inhibitor antibiotics towards S. aureus and MRSA strains used. HPLC-MS-MS analysis through ion trap and subsequent fragmentation of the isolated ions from peels water extracts allowed the clear identification of two well-known phenolic compounds that are known for their antimicrobial activity: Gallic acid (957.6 μg/mL) and Ferulic acid(13 μg/mL).

Conclusion: The ripe fruit of Guava is a nutritionally rich fruit with an outer covering that possess antimicrobial properties.

Key words: Psidium guajava, Peels, Water extracts, MRSA.
Introduction

The continuous emergence of antimicrobial resistant strains of microbes has necessitated the search for novel substances to control their spread. Plants, mostly the leaves and barks, have long been used for the treatment of diseases and infections [1]. However, studies have not focused much on the antimicrobial effects of fruits eaten ripe or unripe. Few studies have indicated the isolation of some antimicrobial substances in fresh fruits and vegetables such as allicin in garlic, eugenol in cloves, hydroxycinnamic acid derivatives (p-coumaric, ferulic, caffeic, chlorogenic acids) in fruits and vegetables [2]. It is of great value to find fruits that are rich nutritionally and have a protective and therapeutic antimicrobial effect as well. Psidium guajava (P.Guava), is an important food crop that is widely used in folk medicine around the world. The fruit is an excellent source of fiber, potassium and vitamin A, in addition to its being free from fat and cholesterol [3]. Various plant parts contain important phytochemicals such as phenolic compounds, flavonoids, carotenoids, terpenoids and triterpenes that possess useful biological and pharmacological activities [3]. Many pharmacological studies have demonstrated the antioxidant, hepatoprotective, anti-allergic, antimicrobial, anti-genotoxic, anti-plasmodial, anti-spasmodic, cardioactive, antitussive, anti-diabetic, anti-inflammatory and anti-nociceptive activities of this plant which supports its traditional uses [3]. Psidium guajava juice has also been used as a hypoglycemic agent[4]. Studies on the antimicrobial effect of guava have been carried mainly on extracts of leaves, shoots and barks, as well as seeds [5,6,7,8,9]such extracts have also been found to inhibit the growth of drug resistant microbes such as V. cholera[10]and to act synergistically with existing antibiotics to treat the pathogenic Staphylococcus aureus [11]as well as other food pathogens [5, 6, 7, 11,12], or in improving oral hygiene and inhibiting biofilm formation by Streptococcus mutant [13].

The present study aims at screening for antimicrobials in the raw ripe fruit. Water extracts are used for their close resemblance to the traditional way of the fruit consumption. Water extracts of the ripe fruit parts are screened for antimicrobial properties.

Materials and Methods

Plant materials

Ripe fruits and leaves of P. guajava obtained from the local market in Amman city- Jordan were used. Fruits and leaves were collected from Jordan valley during the autumn season (September-November 2013). The leaves, aromatic when crushed, are evergreen, opposite, short-petiole, oval or oblong-elliptic, somewhat irregular in outline; 2 3/4 to 6 in (7-15 cm) long, One to two inches (3-5 cm) wide, leathery, with conspicuous parallel veins, and more or less downy on the underside. The fruit, exuding a strong, sweet, musky odor when ripe, may be round, ovoid, or pear-shaped, 2 to 4 inch (5-10 cm) long, with 4 or 5 protruding floral remnants (sepals) at the apex; and thin, light-yellow skin, with an approximate thickness of 0.6-1.2 cm. Next to the skin is a layer of somewhat granular flesh, 1/8 to 1/2 inch (3-12.5 mm) thick, white, yellowish, juicy, subacid, or sweet and flavorful. The central pulp, concolorous or slightly darker in tone, is juicy and normally filled with very hard, yellowish seeds, 1/8 inch (3 min) long. Actual seed counts have ranged from 112 to 535 [14]

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 fruits were peeled off and the thin yellow peel was cut into small pieces. The peeled fresh Guava fruit was then separated into two parts (flesh and seeds) using a clean sharp knife. Each part is homogenized separately using an electrical blender. The homogenates are then freeze dried using a
bench top freeze dryer (HetoFD3 Denmark). Powder of each part is collected in a clean dry container and sealed until use.

**Plant fruit and leaves extraction:**

Water extracts were prepared by reconstituting the lyophilized part of the fruit in distilled water (milligram per milliliter of distilled water), then shaking vigorously for 5 minutes and then allowing it to set at room temperature for 30 minutes. The mixture is then centrifuged at 5000 rpm until complete separation and a clear fluid in the supernatant is obtained. Supernatant is then collected and filtered through a syringe membrane filter (0.45µm). The following concentrations are used for all parts of guava fruit as well as the acetone extracts of leaves: 1%, 2.5%, 5%, 10%, 20%, 35%, and 40%(w/v).

Acetone leaf-extract of Guava was a generous gift from Dr. F. Qadan, Faculty of Pharmacy, Petra University, Amman, Jordan.

**Bacterial Cultures**

American Type Culture Collection (ATCC) and clinical isolates are used. Gram negative bacteria: *E. coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027. Gram Positive bacteria: *Bacillus subtilis* (ATCC) and *Staphylococcus aureus* ATCC 6538. *Propionibacterium acnes* (clinical isolate), Methicillin resistant *S. aureus* (MRSA) (clinical isolates obtained from King Hussein Cancer Center (KHCC), Specialty Hospital and Jordan University Hospital Amman-Jordan), Coagulase negative Staphylococcus (CoNS) and *Propionibacterium acnes* and the yeast *Saccharomyces cerevesea*.

**Clinical isolation of microorganisms**

For the isolation of coagulase-negative *Staphylococcus* the chin area and the face near the nose of volunteers are swabbed using sterile swabs. Swabs are cultured on nutrient agar plates and incubated at 37°C for 24 hours. Colonies obtained are identified using gram stain, growth on Mannitol Salt agar (Himedia, India) and coagulase test. *Propionibacterium acnes* isolated using the method described in Qadan et al., (7). Identification of isolates is performed using colonial morphology, gram stain and the RapID ANAsystem (REMEL-USA).

**Screening for antimicrobial activity**

Muller Hinton agar (MHA) (Himedia India) plates are seeded with bacterial cultures using a sterile swab. Inoculum standardization of culture is done according to the methods described by the clinical laboratory standards institute CLSI (15). Briefly, preparing a suspension from an 18 hours culture that represents 0.5 McFarland standard (approximately 10^8 CFU/ml).

Wells are made aseptically in the seeded agar using the back of a sterile Pasteur pipette. An aliquot (100µl) from each guava extract concentration is applied in the designated well. Control antibiotic discs (ampicillin and amoxicillin for Gram positive bacteria and chloramphenicol for Gram negative bacteria) are placed in the middle of the plate. Plates are incubated at 37°C for 24 hours. At the end of the incubation period the diameters of zones of inhibition are measured in mm.

**Minimal Inhibitory Concentration**

Sterile plant extracts are two fold serially diluted in 1 ml aliquots of Mueller Hinton Broth (MHB) (Himedia, India) starting from 20%. 24-hour-culture of test bacteria in Tryptic soy broth (TSB) (Himedia, India) are adjusted to 0.5 McFarland standard which represents 2x10^8 CFU/ml and added to tubes to reach a cell density of 10^5 CFU/ml. Tubes are incubated at 37°C for 24 hours. MIC is read as the lowest concentration that inhibits bacterial growth as seen by loss of visual turbidity.

**Synergism between extracts and antibiotics**

Betoni et al., [9] methods used with some modification to suit the conditions of the experiment.
Briefly, aliquots of sterile water extracts of peels are added to coolmelted Mueller Hinton Agar (MHA) at ¼ MIC mixed and immediately poured in Petri dishes. Plates are prepared with and without peel extracts. Inoculum size for S. aureus and MRSA strains is adjusted as previously mentioned (2.4) and are seeded on plates using sterile swabs. Antibiotic discs of oxacillin, vancomycin, cefoxitin, and gentamicin are added. Plates are incubated at 37º C for 24 hours. Zones of inhibition are measured in mm. Results presented are the average of triplicate readings.

HPLC-MS-MS analysis
Chromatographic separation of 10% water extracts P. guajava peels is performed on a reversed phase Thermo Scientific™Hypersil™ - Keystone, BDSC8 column (150 x 4 mm, 5 µm) using an Agilent 1200 LC system (Agilent, Santa Clara, CA, USA) equipped with degasser (G1379B), binary pump (G1312A) along with auto sampler (G1367B). The auto sampler is 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 (40:60v/v) was applied at a flow rate of 1.0 ml/min. The column temperature is kept at 27°C. The total analytical run time is 6.0 min for each sample. Detection is carried out on an AB Sciex (Applied Biosystem/MDS SCIEX, Foster City, CA, USA). API-3200 Q-Trap mass spectrometer, equipped with a Turboion spray interface operated in negative ion mode (ESI). Quantification is performed using multiple reactions monitoring (MRM) method. Instrument parameters optimized are 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; source temperature: 550°C. Compound dependent voltage parameters (Declustering potential DP, Enterance potential EP, Collision energy CE, and Collision cell exit potential CXP) are as listed in Table 3. System control and data analysis are performed by AB Sciex Analyst software (version 1.5).

Phenolic compounds identification and quantification
Different phenolic compounds are identified by their MS-MS after ion isolation using linear ion trap in comparison with the corresponding pure standards. The expected phenolic compounds are used based on previous studies [3, 15, 16, 17]. Quantification of the detected phenolic compounds is performed using area under the peak of the isolated ions and the established one point calibration of the corresponding standard compounds. All samples are analyzed in triplicates.

Statistical analysis
Analysis of significance of differences in zones of inhibition of antibiotics in the presence and absence of water extracts of peels is performed using the Mann-Whitney test. P<0.05 was considered significant.

Results
Of the P. guajava fruit parts that are tested in this study, only water extracts of peels showed an antimicrobial effect. At 10% concentrations, coagulase positive S. aureus (CoPS) and MRSA are inhibited with an inhibition zone diameter of 20mm. While concentrations of ≥1% inhibited CoNS with an inhibition zone diameter of 20mm. Acetone extracts of P. guajava leaves had an inhibitory effect on S. aureus at concentrations ≥10% with an inhibition zone diameter of 20mm. Acetone extracts of P. guajava leaves had an inhibitory effect on S. aureus at concentrations ≥10% with an inhibition zone diameter of 10mm and 13mm at 35%. Concentrations 25% and 35% of the leaf extract are inhibitory to CoNS (inhibition zone diameter of 12, 13mm respectively). Concentrations ≥ 25% of acetone leaf extract are inhibitory to the yeast S. cerevesea; with an diameter of inhibition zone of 9mm and 13mm at 35%. Moreover, there is no effect on Gram nega-
Table 1. Antibiotics susceptibility changes of MRSA and S. aureus after exposure to Guava peel water extract.

<table>
<thead>
<tr>
<th></th>
<th>Diameter of Inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRSA</td>
</tr>
<tr>
<td>Extract</td>
<td></td>
</tr>
</tbody>
</table>
| Water extracts of P. guajava peels exerted an anti-MRSA effect at 10% and 20% concentrations with a diameter of inhibition zone of 20mm. Synergism experiments carried further on showed that when MRSA was exposed to ¼ MIC of water extracts of P. guajava peels together with different antibiotics, its susceptibility to vancomycin, oxacillin and cefoxitin is significantly (p=0.034) restored (Table 1). S. aureus exposed to the same conditions showed improved susceptibility towards these antibiotics (Table 1).

Furthermore, oxacillin activity towards 6 different strains of MRSA obtained from different locations was improved (Table 2).

The HPLC-MS-MS (Table 3) analysis with the ion trap at room temperature and subsequent fragmentation of the isolated ions of the water extracts of P. guajava peels allowed the clear identification of two well-known phenolic compounds: Gallic acid (GA) (m/z = 168.8 Da) at a concentration of 957.6 µg/mL appeared at 1.18 min (Figure (a)), and Ferulic acid (FA) (m/z = 192.9 Da) at a much lower concentration (13 µg/mL) (Figure (b)).

Discussion

Results of this study confirm that P. guajava peels possess antimicrobial activity such as peels of pomegranate and apple on both S. aureus and P. fluorescence [18, 19] as well as peels of the Tunisian azarole on S. aureus and S. faecalis [20].

This inhibitory effect was strain and concentration dependent; Gram positive bacteria were more susceptible to the effect of P. guajava peel compared to Gram negative. This effect could be attributed to the structure of gram negative bacterial cell wall that provides a level of intrinsic resistance to certain hydrophilic substances and thus preventing the penetration of active materials in water extracts into the bacterial cell. This could provide an explanation for our results [10,21]. Inhibitory effects of acetone leaf extracts showed similar results to those of other studies [6, 10,22].

Antibiotic resistance is a growing problem that has attracted the attention of researchers to find

Table 2. Synergy between P. guajava peel water extract and Oxacillin against S. aureus strains

<table>
<thead>
<tr>
<th></th>
<th>Diameter of zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>40</td>
</tr>
<tr>
<td>Oxacillin+peel extract</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

S*: Oxacillin sensitive S. aureus; O*: Oxacillin resistant S. aureus
new antibiotics. Improving effectiveness of existing antibiotics is one way of dealing with this problem.

Gallic acid and ferulic acid are known to possess antimicrobial activity against *S. aureus* and other bacteria (*E. coli, P. aeruginosa* and *L. monocytogenes*) [23]. The two phenolic acids cause irreversible changes in bacterial membrane properties (charge, intra and extracellular permeability and physicochemical properties) through hydrophobicity changes, decrease of negative surface charge, and rupturing of cell membranes leading to leakage of essential intracellular constituents [23]. Thus, it seems peculiar that with such mechanism of action for the identified phenolic acids there is no effect against Gram negative bacteria used in this study. The indicated concentrations of the two phenolic acids that resulted in the previously mentioned damage on *S. aureus* are 1750µg/mL and 1250µg/mL for GA and FA respectively [23], which are higher than the concentrations detected in the water extracts of Guava peels. A large difference is observed between the detected MIC of the two phenolic acids in the previous study and the concentrations detected for these two acids in the present study, which indicate higher potencies for these compounds when they are in the crude extracts, rather than as pure single structures. This difference suggests the presence of other substances that contribute to the antimicrobial properties of the water extract of peels, which increases the potency of the identified compounds in their crude extract. Several previous studies found quercetin and its derivatives [3, 15, 21, 24, 25] are responsible for the antibacterial activity in *P. guajava* which were not detected in extracts of this study (Fig. 1). Birdi et al., [24] suggested other constitutes in *P. guajava* such as tannins, flavonoids, saponins and a mixture of diverse volatile compounds to be responsible for the observed antimicrobial activity, rather than quercetin alone. Such substances were not successfully identified by the method of analysis used in this study. However, the current findings emphasize what others have noted that as the *P. guajava* fruit ripens the peels of the fruit would contain higher levels of phenolics than other parts of the fruit [26]. This explains the detected antibacterial activity of the peels compared to other parts of the fruit.

Cefoxitin, vancomycin and oxacillin are cell wall synthesis inhibitor antibiotics. If the antimicrobial effect of the peel extract is to be limited to the presence of gallic and ferulic acids alone, synergism could be explained by membrane effect of these acids that will certainly amplify the role of the three cell wall synthesis inhibitor antibiotics. Interestingly however, a similar synergistic effect was observed between Epigallocatechin Gallate (EGCg) (catechina-

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MS M/Z</th>
<th>MS-MS Fragment M/Z</th>
<th>DP</th>
<th>EP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagic acid</td>
<td>300.6</td>
<td>200.9</td>
<td>-64</td>
<td>-5</td>
<td>-42</td>
<td>-1</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>168.8</td>
<td>124.9</td>
<td>-50</td>
<td>-5</td>
<td>-20</td>
<td>-2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>300.9</td>
<td>150.9</td>
<td>-50</td>
<td>-5</td>
<td>-27</td>
<td>-1</td>
</tr>
<tr>
<td>P-Coumaric acid</td>
<td>162.7</td>
<td>118.8</td>
<td>-33</td>
<td>-5</td>
<td>-20</td>
<td>-2</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>192.9</td>
<td>133.9</td>
<td>-35</td>
<td>-6</td>
<td>-23</td>
<td>-2</td>
</tr>
<tr>
<td>Propylgallate</td>
<td>210.6</td>
<td>124</td>
<td>-60</td>
<td>-5</td>
<td>-35</td>
<td>-2</td>
</tr>
<tr>
<td>Pyro-catechol</td>
<td>108.7</td>
<td>64.8</td>
<td>-63</td>
<td>-5</td>
<td>-37</td>
<td>-1</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>174.9</td>
<td>114.9</td>
<td>-37</td>
<td>-5</td>
<td>-17</td>
<td>-2</td>
</tr>
</tbody>
</table>

Table 3. The expected phenolic compounds MS, MS-MS and dependent voltage parameters

This article is available from: www.iajaa.org / www.medbrary.com
isolated from Camellia sinensis Tea) and carbapenems [27, 28]. EGCg was found to synergize with β-lactams on their effect on MRSA by interfering with the integrity of the cell wall through direct binding to peptidoglycan.

Pelengini et al., [11] extracted P. guajava seeds us-

Figure 1 Intact ion Q1= 168.8 Da and the fragment ion Q3= 124.9 Da for Gallic acid at retention time 1.18 min, the intact ion Q1= 192.9 Da and the fragment ion Q3= 133.9 Da for Ferulic acid at retention time 1.16 min were shown in chromatograms A and B, respectively.
ing 0.6 M NaCl and 0.1% HCl and came up with an antimicrobial peptide that was effective against Gram negative bacteria. In the present study, the seeds were crushed then lyophilized. No attempts were made to extract the seeds using any organic or inorganic chemicals, which, might explain the absence of any antimicrobial effect.

Both coagulase negative staphylococci and coagulase positive S. aureus are increasingly causing clinically significant infections that range between dermatological, respiratory, gastrointestinal, and urinary tract infections [29]. Thus the inhibitory effect of P. guajava peels on staphylococci is worth considering and warrants further investigation.

The results of this study suggest that water extracts of P. guajava peels contain antibacterial compounds that can be used as a natural preservative in food as well as in controlling staphylococcal infections including those caused by the potent pathogen MRSA. These antibacterial compounds acted in synergy with tested cell wall synthesis inhibitor antibiotics.

Acknowledgement

The authors wish to thank Mr. Homam Falaha for his assistance in synergism experiments.

References


This article is available from: www.iajaa.org / www.medbrary.com


