Short Communication

Antibacterial Activity of *Psidium guajava* Leaf and Bark against Multidrug-Resistant *Vibrio cholerae*: Implication for Cholera Control

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SUMMARY: In clinical cholera, a 3-day course of antibiotic complements extensive rehydration therapy by reducing stool volume, shortening the illness, and averting death. However, antibiotic therapy, which has lifesaving implications for cholera, is often hindered due to multidrug resistance in *Vibrio cholerae*, the cause of cholera. Crude aqueous mixture and water soluble methanol extract from leaf and bark of *Psidium guajava*, a tropical fruit guava of the family *Myrtaceae*, showed strong antibacterial activity against multidrug-resistant *V. cholerae* O1. The in vitro minimum inhibitory concentration of the crude aqueous mixture and water soluble methanol extract, which was bactericidal against 10⁷ CFU/mL of *V. cholerae* was determined to be 1,250 µg/mL and 850 µg/mL, respectively. The antibacterial activity of *P. guajava* was stable at 100°C for 15–20 min, suggesting nonprotein nature of the active component. The growth of *V. cholerae* in rice oral rehydration saline (ORS) was completely inhibited when 10 mg/mL (wt/vol) of crude aqueous mixture was premixed with the ORS in a ratio of 1:7 (vol. ex-tract/vol. ORS). *P. guajava*, which is widely distributed in Bangladesh, thus offers great potential for use in indigenous, herbal medicine for controlling epidemics of cholera.

The severe intestinal infection in cholera leads to rapidly progressing dehydration, which without appropriate intervention kills half of affected individuals. Electrolyte replacement therapy through oral and/or intravenous rehydration has proven extraordinarily effective in treating patients from deadly episodes of cholera. In addition, clinically typical cholera patients are routinely treated with 1–3 day courses of antibiotics to shorten the illness (1) and reduce rapid dehydration due to purging (2,3). But, antibiotic therapy in recent years has faced difficulties due to the rapid emergence of multidrug resistance among the cholera bacteria. Since 1977, outbreaks of cholera caused by multidrug-resistant (MDR) *Vibrio cholerae* have been reported in Africa, Asia, and America (4–8). During the past two decades, several cholera endemic countries including India and Bangladesh have reported *V. cholerae* serogroup O1 resistant to tetracycline, ampicillin, kanamycin, streptomycin, sulphonamides, trimethoprim/sulfoxamazole, gentamicin, furazolidone, norfloxacin, ciprofloxacin, and erythromycin (1,9,10). A higher minimum inhibitory concentration (MIC) and clinical failures have pushed clinicians to move to prescribing azithromycin instead of ciprofloxacin, as it is the only drug that is effective for the treatment of cholera at present. Therefore the future effective treatment of cholera is becoming increasingly uncertain.

For centuries, nature has been an enormous source of agents of medical importance. In Nigeria, almost all plants are medicinal and the application of medicinal plants especially in traditional medicine is currently well established as a viable profession (11). *Psidium guajava*, a tropical fruit guava of the family *Myrtaceae*, is widely recognized as a plant of many herbal medicines. The leaf, root, and bark of *P. guajava*, which is widely distributed in Bangladesh, are used in indigenous herbal medicine for the treatment of various ailments including those that are bowel related (12). In the present study, we report the antibacterial activity of *P. guajava* leaf and bark against the MDR, toxigenic *V. cholerae* O1 responsible for epidemic and pandemic cholera outbreaks that kill millions of people worldwide.

Fresh *P. guajava* leaves and bark were washed in water and air-dried at ambient room temperature, which ranged from 26–35°C. The leaves and bark were then dried further in an oven at 60°C for 48 h and crushed into a coarse powder with the help of a mortar and a pestle. The dehydrated powder of *P. guajava* leaf or bark was mixed with sterile distilled water to make a 20 mg/mL (wt/vol) aqueous mixture for antimicrobial assay. For experimental purpose, the antibacterial component from 800 mg of dehydrated leaf powder was extracted with 75% methanol for 48 h. The methanol extract was centrifuged at 12,000 rpm for 10 min, and the supernatant passed through 0.22 µm Millipore membrane filter (Millipore, Bedford, Mass., USA), and dried by rotary vacuum evaporator to remove the residual methanol and water. To this dried preparation, 40 mL of sterile distilled water was added, stirred well and
the water insoluble part was separated again by centrifugation at 12,000 rpm for 10 min. The supernatant was passed through 0.22 μm Millipore membrane filter and dried once again. The recovered dried mass of the water soluble methanol extracted preparation, which weighed 13.6 mg, was dissolved in 1 mL of sterile distilled water to make a stock working solution of 13.6 mg/mL (wt/vol).

To perform the agar diffusion assay, molten Mueller-Hinton (MH) agar was seeded with 10^5 CFU/mL of freshly grown bacterial cells at 45°C. The seeded MH agar was then poured onto a plate and allowed to solidify at room temperature. Wells were prepared on the solid MH agar plates with a sterile borer and the bottoms of the wells (7-mm radius and 6-mm depth) were sealed with molten MH agar (50 μL at 45°C). The crude aqueous mixture or water soluble methanol extract (13.6 mg/mL; wt/vol) were serially diluted (twofold) in Mueller-Hinton broth as diluents. The zone diameters were then measured with slide calipers and the most that inhibited the growth of bacteria completely was considered as the MIC. The MBC was determined by enrichment of the contents of each of the vials (of the MIC experiment) into fresh broth (incubating over night at 37°C), and subculturing onto nutrient agar. The lowest concentration of the crude aqueous mixture or water soluble methanol extract that showed no growth of the bacteria on the agar media was recorded as the MBC for V. cholerae.

Table 1. The minimum inhibitory concentration (MIC) of crude preparation and methanol extract of P. guajava leaf against V. cholerae

<table>
<thead>
<tr>
<th>Serial</th>
<th>V. cholerae O1 strain</th>
<th>Source</th>
<th>Antibacterial resistance pattern</th>
<th>P. guajava leaf</th>
<th>Crude preparation MIC (μg/mL)</th>
<th>Methanol extract MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
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<td>TET® E® SXT® Fr R</td>
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<td>1,250</td>
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<tr>
<td>6</td>
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<td>Fr R</td>
<td></td>
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<td>1,250</td>
</tr>
</tbody>
</table>

1) determined against 10^7 CFU/mL by microdilution method.
2) determined by standard disc diffusion method (9).
3) the crude aqueous mixture (20 mg/mL; wt/vol) and the methanol extract (13.6 mg/mL) were serially diluted (twofold) in Mueller-Hinton broth as diluents.

the water insoluble part was separated again by centrifugation at 12,000 rpm for 10 min. The supernatant was passed through 0.22 μm Millipore membrane filter and dried once again. The recovered dried mass of the water soluble methanol extracted preparation, which weighed 13.6 mg, was dissolved in 1 mL of sterile distilled water to make a stock working solution of 13.6 mg/mL (wt/vol).

V. cholerae O1 strains used in the present study (from the International Center for Diarrhoeal Disease Research, Bangladesh [ICDDR,B] cholera surveillance system), with their sources and resistance patterns to different commonly used antibacterial agents, are shown in Table 1. The antibacterial activity of the crude aqueous mixture (both leaf and bark) or water soluble methanol extract of P. guajava leaf was determined by agar diffusion assay method, as described elsewhere (13).

To perform the agar diffusion assay, molten Mueller-Hinton (MH) agar was seeded with 10^5 CFU/mL of freshly grown bacterial cells at 45°C. The seeded MH agar was then poured onto a plate and allowed to solidify at room temperature. Wells were prepared on the solid MH agar plates with a sterile borer and the bottoms of the wells (7-mm radius and 6-mm depth) were sealed with molten MH agar (50 μL at 45°C). The crude aqueous mixture or water soluble methanol extract (60 μL/well) was then dispensed into each well. After 24 h of incubation at 37°C, all the plates were examined for zones of bacterial growth inhibition. The zone diameters were then measured with slide calipers and the data recorded.

The MIC and minimum bactericidal concentration (MBC) for both the crude aqueous mixture and water soluble methanol extract of P. guajava leaf were determined by microdilution method (14). Precisely, the crude aqueous mixture (20 mg/mL; wt/vol) and the methanol extract (13.6 mg/mL) were serially diluted (twofold), separately, in MH broth (Merck, Darmstadt, Germany) as diluents. The bacterial inoculum was prepared in MH broth so as to give V. cholerae cell density that was determined to be 5.0 × 10^8 CFU/mL. A set of 13 test tubes, each containing 1 mL of both bacterial suspension (5.0 × 10^7 CFU/mL) and different dilutions of either crude aqueous mixture or the methanol extract of guava leaf were incubated at 37°C for 24 h and observed for visible bacterial growth. The lowest concentration (i.e., the highest dilution) of the active ingredient that inhibited the growth of bacteria completely was considered as the MIC.

The MBC was determined by enrichment of the contents of each of the vials (of the MIC experiment) into fresh broth (incubating over night at 37°C), and subculturing onto nutrient agar. The lowest concentration of the crude aqueous mixture or water soluble methanol extract that showed no growth of the bacteria on the agar media was recorded as the MBC for V. cholerae.

The heat stability of the active antibacterial agent in P. guajava leaf and bark was determined by incubating different aliquots of the aqueous mixture and water soluble methanol extract at different temperatures such as 70, 80, or 100°C in a water bath incubator for 30 min. Also, P. guajava leaf or bark powder was boiled in water for 15–20 min. These heat-treated aqueous preparations were then subjected to antibacterial activity assay against V. cholerae cells by the method as described above.

The antibacterial activity of guava leaves in rice oral rehydration saline (ORS), which plays a vital role in saving lives in case of severe dehydration caused by cholera and diarrhea, was tested by the following method. The crude aqueous mixture (20 mg/mL) of dried guava leaves was diluted two-fold in rice ORS and this preparation was then mixed with different proportions of freshly prepared rice ORS. The rice ORSs with or without different proportions of aqueous mixture of P. guajava leaf were then inoculated with 100 μL (5.0 × 10^7 CFU/mL) of V. cholerae cells suspended in sterile rice ORS. The inoculated tubes were then incubated at 37°C overnight and tested for any bacterial growth on a TTGA plate (Difco, Detroit, Mich., USA) after overnight incubation at 37°C.

Now, as the rampant emergence of MDR bacteria is making the future management of infectious diseases uncertain, the World Health Organization (WHO) is putting emphasis on plants as a source of a large number of potential drugs with live-saving implications (15). In this study, the crude aqueous mixtures of both leaf and bark of P. guajava showed equal potential as an antibacterial agent effective against MDR V. cholerae. As shown in Table 1, the MIC of the crude aqueous mixture and water soluble methanol extract of P. guajava
leaf against *V. cholerae* was determined to be 1,250 μg/mL and 850 μg/mL, respectively. *V. cholerae* cells showing no growth in the MIC experiments were enriched overnight transferring the whole contents to fresh LB broth and culturing on solid LB agar media to examine the nature of the antibacterial activity observed in the present study. Results revealed that the *V. cholerae* cells that failed to grow in the presence of the effective dose of the *P. guajava* active ingredient in the MIC experiments did not show any growth response even after prolonged enrichment, confirming that the nature of the antibacterial activity was bactericidal (Table 1); but not bacteriostatic. The bactericidal property of the *P. guajava* demonstrated in the present study may be of advantage from the choleran transmission point of view, given that the *V. cholerae* cells can become noncultivable under certain conditions, without losing their infective potential (16).

The antibacterial activity of the crude aqueous mixture and methanol extract was found to be stable at 100°C, suggesting nonprotein nature of the active compound(s). In addition, the antibacterial activity was released into the aqueous solution when leaf or bark was boiled in water for 15–20 min. Although the active component(s) from *P. guajava* was not isolated to purity in the present study, antibacterial agents extracted and characterized earlier from the leaves and barks of *P. guajava* were shown to be flavonoid in nature (17,18).

In herbal medicines, the active components almost always act in the presence of many other complementary substances that are generally harmless. Such components give better stability and effectiveness to the active component than that of its isolated and pure form (19). The isolation and determination of the pharmacological properties of the active components of herbal remedies allow for better formulations with reduced toxicity, and synthesis of more potent drugs on a commercial scale (20,21). *P. guajava* is the single source of many beneficial components (probably the highest in number) of herbal remedies, which are edible without any known detrimental effect (18). Its wide availability in Bangladesh and effectiveness against cholera bacteria thus make *P. guajava* a very promising alternative to commercial antibiotics that are losing efficacy in the treatment of cholera.

An ideal antidiarrheal agent should have the ability to reverse (i) the increased luminal osmolarity of osmotic diarrhea, (ii) the increased electrolyte secretion of secretory diarrhea, (iii) the decreased electrolyte absorption, and (iv) the deranged intestinal motility that causes a decrease in transit time (22). The antidiarrheal agent in *P. guajava* leaf was shown to inhibit the gastrointestinal release of acetylcholine with quercetin, as a possible mode of action for the recovery from diarrhea (23). Furthermore, *P. guajava* leaf extract was demonstrated to act on electrolytes and water transport in a rat secretory diarrhea model, suggesting its potential as a drug of choice for the rapid recovery from acute watery diarrhea (24). As shown in Table 2, the growth of 10^7 CFU/mL of *V. cholerae* (an infective dose for cholera) was inhibited when 6.8 mg/mL (wt/vol) of crude aqueous mixture of *P. guajava* leaf was mixed with rice ORS in a ratio of 1:6 (v/v) and incubated overnight at 37°C. This result of the in vitro inhibition of the infective dose of *V. cholerae* in rice ORS was significant because *P. guajava* leaf having antidiarrheal and antibacterial activity (17,18) was also shown to be effective for electrolyte and fluid replacement in vivo (24). The boiled *P. guajava* leaf and bark extract in rice ORS thus not only offer an alternative to antibiotics in treating cholera but will also help by complementing the recovery process by performing the electrolyte and fluid replacement function; the combined effect would be to avert death in cases of acute cholera. It is not understood if the vibriocidal activity demonstrated in the present study was conferred by the same active component(s) that showed both antibacterial, and electrolyte and fluid replacement functions reported earlier (17,18,24), or if it was conferred by a different component. Nonetheless, we have proven *P. guajava* to be a potential alternative to the commercial vibriocidal agents for the efficient treatment of cholera that kills many people worldwide.

In Bangladesh, where healthcare facilities are inadequate for the growing population and commercial medicines are more of a dream to the poor, cholera and diarrheal diseases continue to remain a longstanding public health concern that need to be addressed (1). For millions who live in remote villages amid the constant risk of recurrent cholera and diarrhea, the boiling of a few tender guava leaves, which are available in every household, together with rice powder, can be a live-saving solution for preventing cholera related death; but they

<table>
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<tr>
<th>Serial</th>
<th><em>V. cholerae</em> O1 strain</th>
<th>Source</th>
<th>Antimicrobial resistance pattern</th>
<th>Growth response of <em>V. cholerae</em> at different concentrations of sample after boiling with ORS at different ratio (rice ORS/aqueous mixture of guava leaves; v/v)</th>
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10: determined by standard disc diffusion method (9).

2: The MIC for the *V. cholerae* strains in rice ORS was determined to be 1,250 μg/mL.

+ = growth; − = no growth.
Abbreviations are in Table 1.
need to know it.

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REFERENCES