

Research Article**Antibacterial effect of *Psidium guajava* leaves extract on extended spectrum β -lactamase and Metallo- β -lactamase producing uropathogens**

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Abstract

Objective: The emerging problem of antibiotic resistance has driven an urge among scientists to explore alternative treatment methods by pursuing the teachings of traditional medicine. Hence the current study was carried out to investigate the efficacy of *Psidium guajava* leaves extract as a possible alternative source of medicine against drug-resistant pathogens. **Materials and methods:** The leaves extract of *P. guajava* was prepared in ethanol and water using a Soxhlet apparatus and its antibacterial activity were tested against 50 representative isolates of Extended spectrum β -lactamase (ESBL) and Metallo- β -lactamase (MBL) producing uropathogens. Qualitative analysis of the antibacterial activity of *P. guajava* was carried out by well diffusion and agar diffusion method. **Results:** It was found that the ethanol extract of *P. guajava* leaves exhibit considerable antibacterial activity with the observed zones of inhibition in the range of 20-25mm against the test isolates. Water extract of *P. guajava*, on the other hand, showed comparatively smaller zones of inhibition i.e., in the range of 15-18mm. In addition, the ethanol extract of *P. guajava* showed an MBC of 30mg/mL for both ESBL and MBL producers. Furthermore, they showed synergistic activity with ampicillin antibiotic, which was indicated by decreased MBC of ampicillin (i.e., in the range of 200-300 μ g/mL from >10mg/mL) against ESBL and MBL producers in our study. **Conclusion:** Thus, *P. guajava* plant extracts showed promising results to be used as an alternative treatment source for infections caused by ESBL and MBL producing uropathogens.

Keywords: *P. guajava*, extended spectrum β -lactamase, Metallo- β -lactamase, synergy

Introduction

Ever since the discovery of antibiotics, the threat of antimicrobial resistance was predicted by Alexander Fleming. He had stated that there may come a time when penicillin and other future discovered antibiotics may be bought by anyone in the shops. He was worried that the ignorant man will easily take a sub-minimal dose of these antibiotics as supplements which will trigger the development of antibiotic resistance among pathogens (Gallagher, 2015). We are currently living in the future predicted by Alexander Fleming, where there is greater access to antibiotics (Ventola, 2015) and the rampant use of the same in animal husbandry and fisheries (Landers et al., 2012).

The problem of antibiotic resistance appears to be more complex when associated with common infections like urinary

tract infection (UTI) (Torres et al., 2018). The β -lactams are among the most frequently prescribed antibiotics for UTIs due to its efficacy, broad-spectrum activity and low toxicity (Oberoi et al., 2013). Over the years, the indiscriminate use of these antibiotics has consequently resulted in selective pressures among pathogens, generating mutations in the β -lactamase genes which produce resistance that is far more resilient to treatment by current antibiotics. Extended-spectrum β -lactamases (ESBLs) and Metallo- β -lactamases (MBLs) are examples of such selective mutations (Deshmukh et al., 2011). The ESBL producing pathogens are resistant to third generation cephalosporins e.g., cefotaxime, ceftazidime, ceftriaxone etc. In addition to these antibiotics, MBL producers show resistance to last resort carbapenem antibiotics viz., meropenem, ertapenem and imipenem (Giske et al., 2008; Cornaglia et al., 2007). Since UTIs are the second most common infections occurring worldwide, the occurrence of ESBL and MBL genes among uropathogens is especially troublesome because of its associated risk of uncontrolled and easy dissemination in the environment.

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The rising drug resistance urges the need for new drug development programs. However, with the current scenario, it is evident that the new drugs formulated after years of research and huge expenditure will eventually become ineffective (Cassir et al., 2014). It is for this reason that recently the focus of researchers are shifted towards the use of natural products obtained from herbal sources to be used as antimicrobial agents (Borchardt, 2002; Dev, 1999; Cordell and Colvard, 2012).

Psidium guajava, commonly known as guava, is one such plant with known medicinal properties (Gonçalves et al., 2008). It belongs to the Myrtaceae family that also consists of plants like clove, allspice, and eucalyptus whose medicinal properties are well documented (Cascaes et al., 2015; Jamatsing et al., 2018; Dhakad et al., 2018; Harbia et al., 2018). Different parts of *P. guajava* plant have been shown to exhibit therapeutic properties (Abdelrahim et al., 2002; Jaiarj et al., 1999). Traditionally, the leaves of this plant have been useful in overcoming stomach ailments like gastroenteritis, vomiting, diarrhea and dysentery (Jaiarj et al., 1999; Diaz et al., 2017). The essential oils extracted from the leaves of this plant show presence of tannins, terpenes, flavonoids and resin, which together contribute to the antimicrobial properties reported in various literature (Diaz et al., 2017). In addition to the antimicrobial properties, they also exhibit regulating effects on lifestyle disorders like diabetes, hypertension, and obesity (Ncube et al., 2008; Diaz et al., 2017). The leaves of *P. guajava* are also known to be helpful in managing the symptoms of malaria (Bijauliya et al., 2018). The roots and fruit of this plant are effective in the prevention of and treatment of diarrhea (Barbalho et al., 2012). Apart from the above-mentioned disorders, the plant is also useful in treating wounds, cough, toothache, sore throat and inflamed gums (Diaz et al., 2017). In some parts of the world, the wood-stick obtained from the plant is used as “miswak” for oral care (Sanda et al., 2011).

Considering the increasing antibiotic resistance among pathogens, we investigated the antibacterial as well as resistance reversal activity of *Psidium guajava* leaves extract against Extended spectrum β -lactamase (ESBL) and Metallo- β -lactamase (MBL) producing uropathogens.

Materials and methods

Test organisms

Gram-negative pathogens isolated and characterized for ESBL and MBL production in a previous study was used as test organisms (Aruna and Tariq, 2012; Tariq and Aruna, 2015; Tariq and Aruna, 2016). Fifty representative ESBL and MBL producing pathogens including *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *Proteus mirabilis* and *Citrobacter diversus* were used in the current study. These isolates were maintained on Nutrient Agar (NA) slants supplemented with 100 μ g/ml of

ampicillin and stored at refrigerated conditions.

Plant material

Psidium guajava (Guava) leaves were obtained from a local garden and authenticated by an expert botanist from Department of Botany Wilson College, Mumbai.

Preparation of plant extract

The leaves of *Psidium guajava* were washed with distilled water, dried in shade for 10 days, and ground to fine powder with the help of a mechanical blender. The extraction of bioactive compounds was carried out, from a 100g sample in 200mL ethanol using soxhlet apparatus, over a period of 12h. The extract was further concentrated at 40°C on a water bath to obtain a semisolid mass. This mass was re-suspended in ethanol to get the required concentration of the extract for carrying out further analysis. These concentrates were prepared in large volumes and stored at 4°C until further use, in order to avoid batch to batch variations in our study. Water extract of *P. guajava* was also prepared by boiling the leaves in distilled water for 15mins.

Sterility testing of plant extract

The sterility of the extracts was confirmed by checking for bacterial or fungal growth after spot inoculating them on a sterile Nutrient Agar (NA) and Sabouraud's Agar (SAB) plate respectively (Sule and Agbabiaka, 2008). The NA plates were incubated at 37°C and SAB plates at 30°C for an extended duration of 7 days to confirm the absence of contaminants.

Qualitative study of the inhibitory activity of *P. guajava* ethanolic leaves extract against test organisms

The antibacterial effect of *P. guajava* ethanolic leaves extract against test pathogens was determined by agar well diffusion method (Shareef, 2011). Sterile molten NA butt was seeded with 0.4ml of 24h old test pathogens (0.1 OD_{540nm}) and poured into sterile petri-plates. After solidification, wells were punched into the medium using a sterile cork-borer and 50 μ l of plant extracts were added to the same. It was then allowed to diffuse through the wells during its incubation at 37°C for 24h, after which the resulting zones of inhibition were measured. Control wells were also set up using 50 μ l of ethanol (solvent) for each isolate.

Determination of MBC of *P. guajava* ethanolic leaves extract against test pathogens

Agar dilution method was carried out to determine the Minimum Bactericidal Concentration (MBC) of *P. guajava* ethanolic leaves extract against test pathogens. Different concentrations of ethanol extract ranging from 5mg/mL to

50 mg/mL with an interval of 5 mg/mL were supplemented into molten NA butts cooled to around 40°C. The test isolates were spot inoculated (5µL) on solidified medium and incubated at 37°C for 24h. MBC was defined as the lowest concentration of *P. guajava* ethanolic leaves extract that completely inhibited the growth of test cultures (CLSI, 2006).

Evaluation of Synergistic effect by agar dilution method

The agar dilution method was similarly used to determine the synergistic activity between *P. guajava* leaves extract and ampicillin. It was carried out by incorporating sub-lethal (½MBC) concentrations of *P. guajava* leaves extract into molten NA butt which were cooled to around 40°C along with 100-500 µg/mL of ampicillin with an interval of 100µg/mL (CLSI, 2006).

Gas Chromatography-Mass Spectrophotometry analysis

The bioactive components from *P. guajava* leaves extract were analyzed by GC-MS HP 7890 system (Agilent technologies). Capillary column with dimensions 30m X 0.25mm X 0.25µm was equipped. The program used for GC oven temperature was 5min isothermal at 300°C, followed by 90°-260°C at a rate of 10°C/min, then held at 260°C for 5 min. The injection port temperature was 240°C. Along with that a Joel, AccuTOF GCV MS system, with a time of flight analyzer, was used (Indian Pharmacopoeia, 2007). The entire analysis was carried out at IIT Bombay, Mumbai 400076. The compounds of the *P. guajava* leaves extract were identified by comparison of their retention indices (RI) and mass spectra fragmentation with those on the stored library available with IIT, Bombay.

Statistical analysis

All experiments were carried out in triplicates and represented as

mean values.

Results and discussion

Sterility testing of solvent extracts

The extracts of *P. guajava* leaves showed absence of bacterial and fungal contaminants even after 7 days of incubation. The extended incubation time confirmed the absence of slow-growing contaminants and stressed cells that may have survived the processing of solvent extracts.

Qualitative study of the inhibitory activity of *P. guajava* ethanolic leaves extract against test organisms

Table 1 represents the zones of inhibition of solvent extracts of *P. guajava* against ESBL and MBL producers respectively. Water extracts of *P. guajava* showed zone sizes in the range of 15-18mm. Ethanol extracts of *P. guajava*, on the other hand, showed comparatively larger zones of inhibition i.e., in the range of 18-25mm, hence indicating more efficient bioactivity.

A Soxhlet apparatus, invented in 1879 by Franz von Soxhlet, is the most convenient extraction instrument used by researchers to extract and study the efficacy of various biomolecules (Soxhlet, 1879; Redfern et al., 2014). It allows efficient extraction of biomolecules like lipids and essential oil using small amounts of a suitable solvent, by recycling, to obtain a concentrated mixture of desired compound (Redfern et al., 2014). The antibacterial activity of various herbs extracted by using soxhlet apparatus has been determined in the past by several researchers (Redfern et al., 2014; Abeysinghe, 2010; Jeyaseelan et al., 2012). In a similar study, the methanol extracts of *P. guajava* showed

Table 1. Antibacterial activity of *P. guajava* extracts against ESBL and MBL producers

Test organisms	No. of isolates	Zones of inhibition of solvent extracts in mm		Mean MBC in mg/mL
		Water	Ethanol	Ethanol extract
ESBL producers				
<i>Klebsiella pneumoniae</i>	05	16.6±0.47	23.6±0.47	30
<i>Escherichia coli</i>	05	17.3±0.47	24±0.81	30
<i>Pseudomonas aeruginosa</i>	05	16.6±0.47	18.3±1.24	30
<i>Proteus mirabilis</i>	05	16.6±1.24	23±0	30
<i>Citrobacter diversus</i>	05	16.6±0.47	24±0.81	30
MBL producers				
<i>Klebsiella pneumoniae</i>	05	17.6±0.94	21.3±0.94	30
<i>Escherichia coli</i>	05	16.0±0.81	24±0.81	30
<i>Pseudomonas aeruginosa</i>	05	16.6±0.47	23.6±0.47	30
<i>Proteus mirabilis</i>	05	15.6±0.94	22.6±1.69	30
<i>Citrobacter diversus</i>	05	15.6±0.47	22.3±1.69	30

zones of inhibition of 8.27mm and 12.30mm against *B. cereus* and *S. aureus*, respectively. They also reported comparatively smaller zones of inhibition i.e., 6.11mm and 11.00mm against ethanol extracts of *P. guajava* for *B. cereus* and *S. aureus* respectively (Biswas et al., 2013). *P. guajava* extracts are also well known for their efficacy in oral hygiene (Chandra et al., 2015). In another study, the ethanolic extract *P. guajava* was found to be most effective at 25mg concentration against *P. aeruginosa* (9.50mm), *E. coli* (9.00mm), *S. pneumoniae* (10.50mm) and *K. pneumoniae* (9.50mm). However, the aqueous extract of *P. guajava* was found to be effective only at 100mg concentration inhibiting *E. coli* (12.50mm), *S. aureus* (14.50mm) and *S. pneumoniae* (9.00mm) (Kenneth et al., 2017).

Determination of MBC of *P. guajava* ethanolic leaves extract against test pathogens

Table 1 represents the MBC of the solvent extracts of *P. guajava* against ESBL and MBL producers respectively. For water extract of leaves of *P. guajava* growth was observed up to 100mg/ml concentration of extract. Hence due to experimental limitations, the exact MBC of water extract of leaves of *P. guajava* could not be determined. Ethanol extract of leaves of *P. guajava* showed an MBC of 30mg/ml for both ESBL and MBL producers. A similar study carried out using 12 Philippine medicinal plants (including *P. guajava*) to evaluate their antibacterial activity against multi-drug resistant isolates viz., Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains, Vancomycin-resistant Enterococci (VRE) strains, ESBL and MBL producers, showed their MBC to be in the range of 312-625µg/mL (Demetrio et al., 2015). Another study carried out using 5 different plant extracts and honey sample showed their MBC in the range of 0.625-5 mg/mL. In their study, the best activity was obtained using methanol

extracts of *P. guajava* against test isolates when compared to other 4 medicinal plants (Ramalivhana et al., 2014).

The differences observed in the zones of inhibition and MBC values in our studies when compared to others can be attributed to several underlying factors which cannot be controlled by researchers in certain cases. These factors include the biochemical composition of the plant, soil and growth condition, extraction time, choice of solvents, type of media and inoculum size used among others. Hence, the same studies carried out in two different parts of the world having different environmental conditions may result in different outcomes.

Evaluation of Synergistic effect by agar dilution method

Data shown in table 2 represents the synergistic effect of ethanol extract of leaves of *P. guajava* and ampicillin against ESBL and MBL producers. Interestingly, the MBC value of ampicillin was found to reduce from 10mg/ml to 300-500µg/ml when used in combination with ethanol extract of leaves of *P. guajava*.

Our previous studies carried out with *Trachyspermum ammi* (Tariq et al., 2014), *Ocimum basilicum* (Gore et al., 2015) and *Camellia sinensis* (Tariq et al., 2015) also showed similar results. A recent study also showed synergistic activity between stem bark extracts of *Faidherbia albida* and *P. guajava*, and the MBC of this combination was determined to be 0.5mg/mL against MRSA strains (Tawfiq et al., 2017). Another study was carried out to determine the synergistic activity between the crude extracts of *Cocos nucifera* and six antibiotics viz., ampicillin, penicillin G, amoxicillin, chloramphenicol, ciprofloxacin and tetracycline, against bacterial pathogens. In their study, the

Table 2. Synergistic activity of *P. guajava* leaf extract and ampicillin

Test Pathogens	MBC of Ampicillin	MBC of <i>P. guajava</i> extract	Sub-lethal concentration of <i>P. guajava</i> extract used	Synergy observed-MBC of ampicillin in presence of <i>P. guajava</i> extract
ESBL producers				
<i>Klebsiella pneumoniae</i>	More than 10mg/mL	30mg/mL	15mg/mL	300 µg/mL
<i>Escherichia coli</i>	10mg/mL			400 µg/mL
<i>Pseudomonas aeruginosa</i>				300 µg/mL
<i>Proteus mirabilis</i>				300 µg/mL
<i>Citrobacter diversus</i>				200 µg/mL
MBL producers				
<i>Klebsiella pneumoniae</i>	More than 10mg/ml	30mg/ml	15mg/ml	200 µg/mL
<i>Escherichia coli</i>	10mg/ml			300 µg/mL
<i>Pseudomonas aeruginosa</i>				400 µg/mL
<i>Proteus mirabilis</i>				400 µg/mL
<i>Citrobacter diversus</i>				200 µg/mL

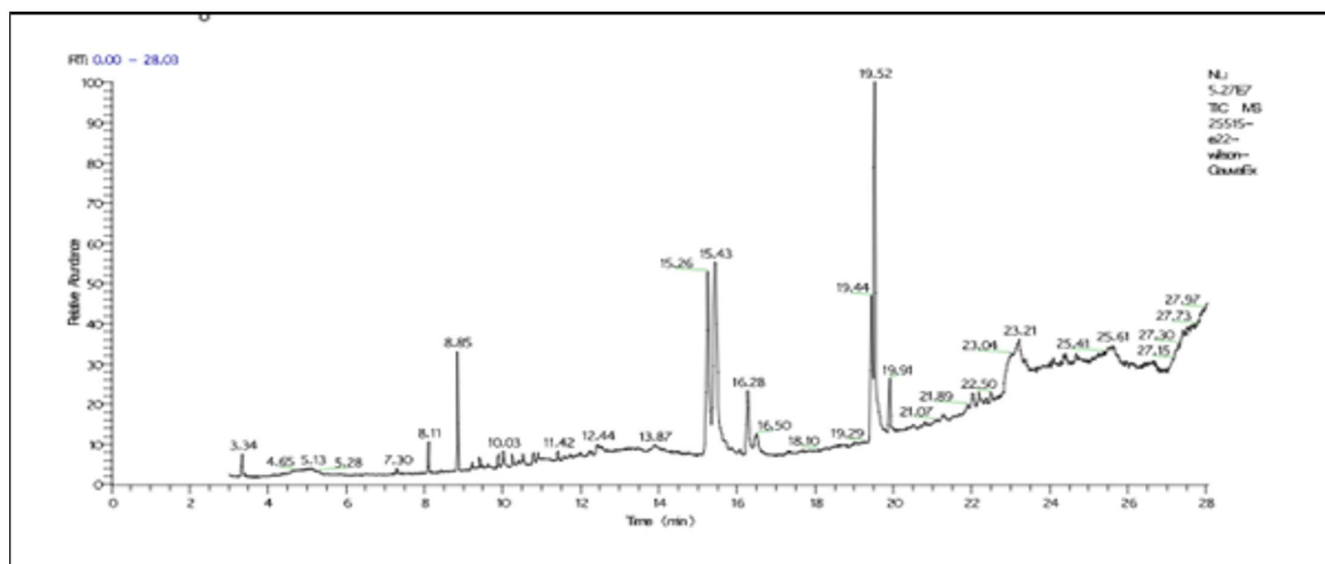


Figure 1. GC-MS chromatogram of *P. guajava*

Table 3. Identified components from GC-MS chromatogram of *P. guajava*

Scientific name	Common name	Retention time	Molecular mass
4,11-Trimethyl -8-methylene undecanoic acid	Caryophyllene	3.34	204
9,12-octadecenoic acid	Linoleic acid	19.44	280
Heptadecenoic acid	Margaric acid	19.91	268
10-octadecenoic acid	Isooleic acid	15.43	282
9,12-octadecenoic acid	Linoleic acid	8.85	280

checkerboard method revealed synergistic interaction in 67% of the combinations, and indifference in 33% (Akinyele et al., 2017). It is well known that antibiotic resistance is on a rise, with pathogens developing resistance to most of the known antibiotics including the newer forms. Hence, the studies that focus on the synergistic approach of treatment methods by reversing the antibiotic resistance is much more valuable given the demand of the current era.

Gas Chromatography-Mass Spectrophotometry analysis

The chromatogram showing Retention Time (RT_m) of several constituents identified with the help of GC-MS analysis of a crude ethanolic extract of leaves of *P. guajava* leaves is shown in figure 1. It showed 5 distinct peaks. The bioactive compounds corresponding to these peaks are provided in table 3. It showed the presence of caryophyllene in small amounts as compared to other unsaturated fatty acids identified viz., Linoleic acid, margaric acid and iso-oleic acid. Beta-caryophyllene is known to initiate TN-F α -induced apoptosis and bacterial invasion by down-modulation of NF-k β regulated gene product. Other functions of caryophyllene include apoptosis, suppression of tumor growth, and inhibition of metastasis (Kim et al., 2014). The high content of unsaturated fatty acids in *P. guajava* leaves extract may be responsible for the antibiotic and synergistic

activity observed in our study (Choi et al., 2013). It is also responsible for increased immunity and cardiovascular health (Fritsche, 2006; Ander et al., 2003). In another study, the result of GC-MS of the methanol extract of the leaves of guava showed 41 compounds. In their study, the chromatogram showed an abundance of Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl) detected at the retention time of 20.837mins, followed by Cyclohexene, 3-(1, 5-dimethyl- 4-hexenyl)-6-methylene at the retention time of 21.299mins (Nwanneka et al., 2013).

Conclusion

The ethanolic extract of *P. guajava* leaves showed significant activity against ESBL and MBL producing uropathogens. Further, its potency can be evaluated against pathogenic drug-resistant bacteria causing other infections. Moreover, the ability of crude extracts to give such promising antibacterial results only enhances the chances of it becoming an important alternate remedy towards the treatment of infections caused by multi-drug resistant pathogens.

Conflicts of interest: Not declared.

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