

## Effect of *Psidium guajava* L. on Biofilm Forming Multidrug Resistant Extended Spectrum Beta Lactamase (ESBL) Producing *Pseudomonas aeruginosa*

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### Abstract

*Psidium guajava* L. (Guava) commonly known for its food and nutritional values. The medicinal properties of leave of *Psidium guajava* is well known in traditional system of medicine. The relative efficacy of guava leave ethanolic extract and guava leave tea and antibiotic standards was tested against pathogenic *Pseudomonas aeruginosa*. This study was carried out at Microbiology laboratory of KIST medical college and teaching hospital, Lalitpur, a tertiary care hospital from 15<sup>th</sup> June 2017 to 15<sup>th</sup> Dec 2017. The isolates were tested for antibiotic susceptibility by Kirby-Bauer disk diffusion method. Biofilm formation was detected by microtitre culture plate method and ESBL production by combine disk methods. Antimicrobial activity of guava leaves were determined by well diffusion method. Ethanol extract of fresh guava leaves exhibited higher antibacterial activity than dry and fresh leaves tea, but significantly less than the standard antibiotics. In this study, 83.67% of biofilm producer, 65.30% of MDR and 6.12% of ESBL producing *P. aeruginosa* were isolated. The leaves extract of guava have shown effective result against *P. aeruginosa* and could serve as good source of antibacterial agents. Guava leaves extract can be an economical ternative to antibiotics. However, active compound of this extract need to be purified and pharmacologically tested before its application.

**Keywords:** Antibacterial activity, Antimicrobial susceptibility testing, Guava

### Introduction

Infectious diseases are the major cause of death in the developing countries and account upto 50% of it. The global burdens of infectious disease are reduced by using antimicrobial agents. However, emergence and spread of resistant pathogen has diminished the effectiveness of the antibiotics (Bisht & Agrawal 2016). *Pseudomonas aeruginosa* is a Gram-negative aerobic and facultative anaerobic bacilli of Pseudomonadaceae family. *P. aeruginosa* is causative agent of various infections such as: urinary tract infections, respiratory infections, otitis media, skin and soft tissue infections, bone and joint infections; and bacteremia. Besides, it can also cause serious systemic infections particularly in people with compromised immune systems including patients of burn suffer, cystic fibrosis, cancer and AIDS (Neopane et al., 2017). It has emerged as one of the leading causes of nosocomial infections. *P. aeruginosa* is the sixth most frequently isolated nosocomial pathogen, causing 7.3% of all hospital

acquired infection in the US (Weiner et al., 2016). Infections due to *P. aeruginosa* are difficult to eradicate due to their intrinsic resistance as well as their ability to acquire resistance to different antibiotics. The resistance is due to over expression of efflux pump, acquisition of extended spectrum  $\beta$ -Lactamases (ESBLs) and Metallo- $\beta$ -Lactamases (MBLs), target site or outer membrane modification, porin mutations and plasmid enzymatic modification (Heydari & Eftekhari, 2015). *P. aeruginosa* can form biofilms, which exponentially increase antibiotic resistance. The three exo-polysaccharides that mainly contribute to the biofilm formation in this bacteria are; alginate, Psl (Polysaccharides Synthesis Locus), and Pel (Pellicle). Alginate confers additional protection against antimicrobials and the immune system while Psl and Pel contribute to aggregation and adherence (Nithyalakshmi et al., 2015). Hence, dealing with multi-drug resistant strain of these bacteria is challenging.

Various types of plants contain natural preservatives which have antimicrobial and antioxidant property.

Guava leave (*Psidium guajava* L), a phyto-therapeutic plant used in folk medicine, is believed to have active components which can be used to treat and cure various diseases (Mailoa et al., 2014). Different parts of the plant have been used in traditional medicine against ailments like malaria, gastroenteritis, vomiting, diarrhoea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, etc. Besides, it has also been used for the controlling of diabetes, hypertension and obesity (Biswas et al., 2013).

In Nepal, study has shown the emergence of multidrug resistant (MDR) isolates of *P. aeruginosa* associated with nosocomial infections. The occurrence of biofilm and MDR bacteria in a hospital setting possess a therapeutic problem, as well as a serious concern for infection control management. Therefore minimizing the use of antibiotics and where possible substituting with antimicrobial compound from other source can be an alternative to subside the growing antibiotic resistant problem. Guava leaf tea and extract from it with antimicrobial activity can be a choice. This study was therefore undertaken to evaluate the antimicrobial activity of guava leaves on *P. aeruginosa* isolated from various clinical samples.

## Materials and Methods

*P. aeruginosa* isolated from different clinical samples such as pus/wound, blood, sputum and urine were identified by standard microbiological techniques. The isolates were further tested for antibiotic susceptibility by Kirby-Bauer disk diffusion method on Mueller Hinton agar as per CLSI guidelines. ESBL production was screened using two disks, ceftazidime (30 µg) and cefotaxime (30 µg) according to the CLSI guidelines. An inhibition zone of  $d \leq 22$  mm for ceftazidime and  $d \leq 27$  mm for cefotaxime indicated as ESBL producing strain which was further confirmed by combination disc method.

## Detection of biofilm production

Biofilm production was detected by microtitre culture plate method (TCP), a quantitative test as

described by Christensen et al. (1995). Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose and were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Sterile 96 flat bottomed polystyrene tissue culture plates were filled with 200 µL of the diluted cultures in each well. The control organisms used was *P. aeruginosa* ATCC 27853. For negative controls wells contained sterile broth without inoculum. The plates were incubated at 37°C for 24 hr. After incubation, contents of each wells were removed by gentle tapping. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) for four times to remove free floating bacteria. Biofilm formed by bacteria in the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained biofilm was obtained by using micro ELISA autoreader at wavelength 570 nm. The experiment was performed in triplicate with three repetition. Interpretation of biofilm production was done according to the criteria explained by Stepanovic et al. (2000).

## Preparation of guava tea and ethanol extract

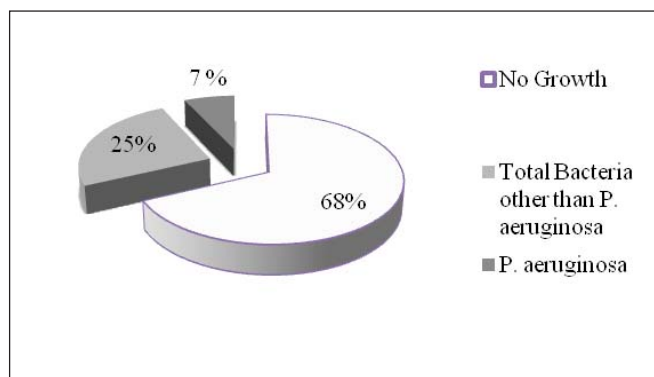
Guava leaf samples were randomly collected from Kathmandu Valley. The leaves were washed with sterile distilled water. Extract first (fresh leaf tea) was prepared (10 leaves were taken in conical flask containing 200 ml of distilled water and boiled for 10-20 minutes). Extract second (dry leaf tea) was prepared from freeze dried leaves of the same weight and numbers. Similarly tea was prepared by same procedure. Extract third and fourth (ethanol extract of fresh and dried leaves respectively) were prepared by using absolute ethanol as a solvent. The leaf pieces were added to solvent. The mixtures were made in sterile flask wrapped in aluminum foil to avoid evaporation and exposed to light for 3 days at room temperature. The flasks were placed on a platform shaker at 70 rpm. After 3 days of soaking in solvent, the mixtures were transferred to tubes and centrifuged for 10 min at 4,000 rpm at 25°C. The supernatant was collected and stored at 4°C until use.

### Determination of antimicrobial activity of guava leave tea against *P. aeruginosa*

Antimicrobial susceptibility testing was done using agar diffusion method in triplicate according to the National Committee for Clinical Laboratory Standards guidelines. The plant extracts were tested on Mueller Hinton Agar (MHA) plates against *P. aeruginosa* to detect the antibacterial activity. Prior to streaking the plates with bacteria, 5mm diameter wells were punched into the medium using a sterile borer. All plates were inoculated with the broth culture of isolated *P. aeruginosa* which has been previously adjusted to a 0.5 McFarland standard solution. A sterile cotton swab was dipped into the bacterial suspension, rotated several times, and pressed firmly to the inside wall of the tube above the fluid level removing excess inoculum. The surface of the agar plate was streaked over the entire sterile agar surface rotating the plate to ensuring even distribution of inoculum. The plates are allowed stand for 5min to absorb the excess moisture content. Exactly 100µl aliquots of each test extract and leave tea were dispensed into each well of MHA plates swabbed with bacteria. For positive control an antibiotic tobramycin was placed at the centre of the plate. While ethanol was used for negative control. *P. aeruginosa* ATCC 27853 was used for positive control organism.

### Results and Discussion

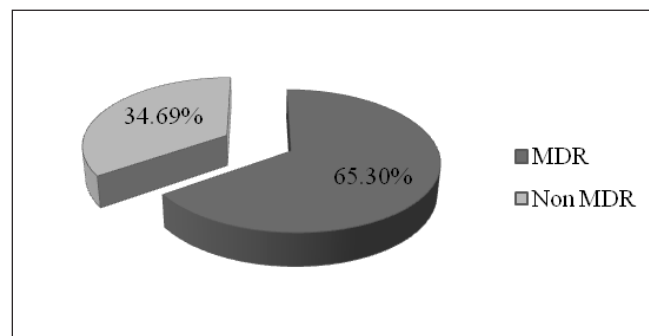
A total of 3000 specimens were processed and bacterial growth was observed in 25% samples and *P. aeruginosa* was isolated from 7% of the total sample (Figure 1)



**Figure 1:** Growth pattern of bacterial isolates in clinical samples

### Prevalence of MDR *P. aeruginosa*

Among the total *P. aeruginosa* isolates 65.3% ( $n=32$ ) were MDR (Figure 2).



**Figure 2:** Percentage of MDR strain from the total isolates of *P. aeruginosa*

### Antibiotic susceptibility pattern of *P. aeruginosa* isolates

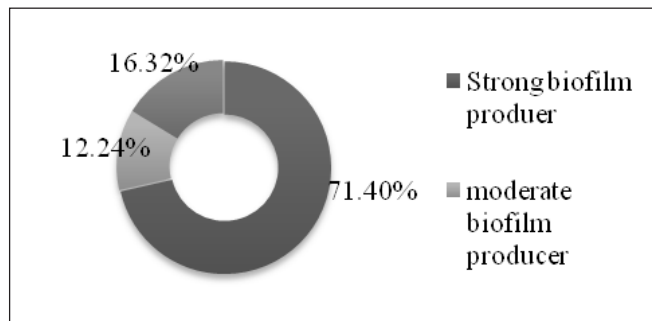
Most of the isolates were sensitive towards colistin and resistant towards cefepime antibiotics (Table 1).

**Table 1:** Antibiotic susceptibility pattern of *P. aeruginosa*

Antibiotic	Sensitive%	Intermediate%	Resistant%
Piperacillin	31(63.3)	-	18 (36.7)
Piperacillin/Tazobactam	27 (55.1)	3 (6.1)	19 (38.8)
Ceftriaxone	20 (40.8)	7 (14.3)	22(46.9)
Ceftazidime	22(44.9)	4(8.2)	23(46.9)
Cefepime	16(32.7)	-	33(67.3)
Aztreonam	29(59.2)	6(12.2)	14(28.6)
Gentamicin	29(59.2)	-	20(40.8)
Amikacin	24(49.0)	1(2.0)	24(49.0)
Imipenem	19(38.8)	1(2.0)	29(59.2)
Meropenem	24(49.0)	2(4.1)	23(46.9)
Ciprofloxacin	30(61.0)	-	19(38.8)
Colistin	46(93.9)	-	3(6.1)
Polymyxin-B	32(65.3)	5(10.2)	12(24.5)
Cefotaxime	20(40.8)	3(6.1)	26(53.1)
Tobramycin	29(59.2)	1(2.0)	19(38.8)

### Biofilm production detection in *P. aeruginosa*

Out of 49 isolates of *P. aeruginosa* 35(71.40%) were strong biofilm producer, while 6(12.24%) were weak producers and 8(16.32%) were non-biofilm producers (Figure 3).



**Figure 3:** Percentage of biofilm producing *P. aeruginosa*

### ESBL production in *P. aeruginosa*

Among the total *P. aeruginosa* isolates, 3 (6.12%) were found to be ESBL producer. Significant numbers of biofilm producers are MDR isolates ( $p < 0.05$ ) (Table 2).

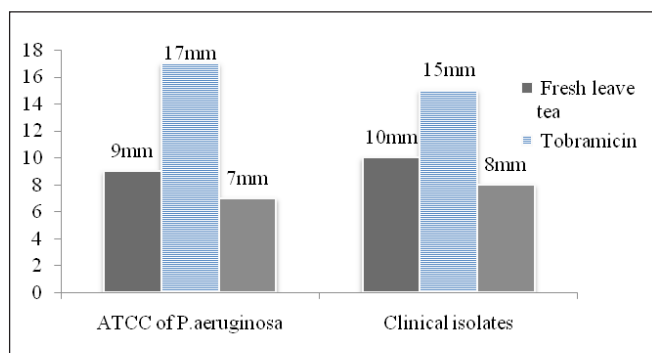
**Table 2:** Comparison between MDR and biofilm production

MDR	Biofilm producer		Total (%)	p-value
	Positive (%)	Negative (%)		
Positive	31 (96.8)	1 (3.2)	32 (100.0)	0.02
Negative	10 (58.82)	7 (41.18)	17 (100.0)	(<0.05)
<b>Total</b>	<b>41 (83.67)</b>	<b>8 (16.33)</b>	<b>49 (100.0)</b>	

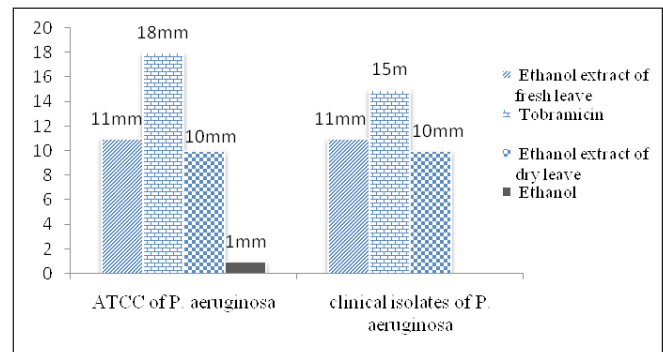
### Antimicrobial activity of guava leaf

Guava leaf tea (fresh and dry leaf tea) showed its antimicrobial activity against all types of *P. aeruginosa* isolates. However, compared to the antimicrobial activity of standard antibiotic tobramycin, the zone of inhibition shown by guava leaf was less (Figure 4). The inhibition zone given by fresh leaf for both guava leaf tea and ethanol extract were higher compared to the dry leaf (Figure 4 and 5).

*P. aeruginosa* is an important opportunistic nosocomial pathogen of great important due to its



**Figure 4:** Antimicrobial activity of guava leaf tea and tobramycin against *P. aeruginosa*



**Figure 5:** Antimicrobial activity of ethanol extract of guava leaves and tobramycin against *P. aeruginosa*

resistance to multiple antibiotics (Goel et al., 2013). In this study, prevalence of *P. aeruginosa* from different clinical samples was found to be 27.22% which is less compared to Ali et al., (2015) and Goel et al., (2013) who reported 39% and 37.7% respectively from Pakistan and India.

High prevalence of MDR *P. aeruginosa* (65.3%) was reported in the study of Fatima et al., (2012) where MDR isolates accounted for 73.9%. This finding is also corroborated with MDR *P. aeruginosa* in Southeast Asia, where 71% reported during 2007-2009 (Suwantararat & Carrol 2016). A high prevalence of MDR *P. aeruginosa* seen in hospital acquired infections was due to selective pressure exerted by over usage of broad spectrum antibiotics. However, the emergence of MDR is related to the empirical use of antibiotics rather than the rational use of broad-spectrum antibiotics before the sample collection.

Prevalence of ESBL producing *P. aeruginosa* was 6.12%. Among the ESBL producer 66.67% were MDR and 6.25% MDR were ESBL producer, which is similar to a study by Stepanovic et al. (2000). But different to Shaikh et al. (2015) who reported 25.13% isolates of *P. aeruginosa* were observed as ESBL positive among the 187 samples. The ESBL producing *P. aeruginosa* isolates exhibited co-resistance against most of the antibiotics tested.

In this study, 83.67% isolates of *P. aeruginosa* were biofilm producer. Maita and Boonbumrung (2014) reported 79.4% biofilm producer in Thailand. A little higher 89.3% was reported by Sharma and Chaudhary (2015) but lower (48.8%) by

Tamaraiselvi et al. (2015). The biofilm production was found to be independent of the antibiotic susceptibility profile of the bacteria. When the degree of adhesion of the biofilm is high, the penetration of the antimicrobial compound into its structure is reduced resulting in the increased resistance of the bacteria.

Antibacterial activity of ethanol extract of fresh leave showed higher activity (11mm) followed by ethanol extract of dry leaves and least activity by dry guava tea solution (7mm). In Philippines, Cruzada et al. (2014) did quantification of biofilm in microtiter plates for overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. Different concentration of guava extract exhibited antibacterial strengths against both, *E. coli* and *P. aeruginosa* bacteria but was significantly less than the standard antibiotics. Biswas et al. (2013) has reported that *P. guajava* has antibacterial effect against both Gram-negative and Gram-positive bacteria. It was due to presence of alkaloids, flavonoids, tannins, saponins, glycosides and terpenoids in the leaves extracts of *P. guajava* (Savoia, 2012). These phytochemicals have in vitro inhibitory activity against some clinical bacterial isolates. In Brazil, Sanches et al. (2005) reported that the aqueous extracts of *P. guajava* leaves, roots and stem bark were active against the Gram positive bacteria but not against Gramnegative species. This can be due to the outer membrane of Gram negative bacteria which act as barrier to penetration of numerous antibiotic molecules. Besides, the enzymes present in the periplasmic space have ability to break down foreign molecules.

## Conclusion

*P. aeruginosa* is a pathogen of interest in most of the hospital acquired infection. Increase in the drug resistant *P. aeruginosa* is a great challenge in treatment of infections caused by it. Since crude ethanol extract of guava leave and its tea was able to inhibit *P. aeruginosa*. The compound present in guava leave extract and its tea contain some antimicrobial compounds which can effectively

control pathogenic bacteria. It has potential for use in therapy against infections caused by pathogens. Thus can be recommended additional test of pure extracts along with further pharmacological evaluation is needed.

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