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Research Article

Antimicrobial Effect of *Psidium guajava* L. Leave Extract in Correlation with Biofilm Formation and Metallo-β-Lactamase Production in Multidrug Resistant *Pseudomonas aeruginosa*

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Abstract: This study was aimed to determine antibacterial effect of *P. guajava* leave extracts and correlation of metallo-β-lactamase (MBL) production and biofilm formation with MDR *P. aeruginosa* isolated from different clinical samples. The study was carried out in the Kathmandu Institute of Science and Technology (KIST) medical college and teaching hospital and Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal. A total of 45 isolates of *P. aeruginosa*, isolated from different clinical samples were identified by standard microbiological techniques and antimicrobial susceptibility of the isolates was tested by Kirby-Bauer disk diffusion method on Muller Hinton agar as per CLSI guidelines. The ability to form biofilm was detected using the microtiter plate assay. MBL production was screened by Imipenem disk diffusion method and confirmed by Imipenem-EDTA combined disk method. P. guajava leave extracts were prepared using absolute methanol and hydroethanol solvent at different ratios. The antimicrobial activity of *P. guajava* leave extract against the pseudomonal isolates was determined by agar well diffusion method. Out of 45 isolates of *P. aeruginosa*, 30 (67%) were multidrug resistant (MDR) isolates, 30 (67%) were biofilm producers and 6 (13%) were metallo β lactamase (MBL) producers respectively. The methanol extract of fresh P. guajava leave (13mm) showed higher activity and least activity by 7:3 hydroethanol extract of dried *P. guajava* leave (6mm) toward the *P. aeruginosa* isolates. The methanol extract may be an alternative source for Pseudomonal infection treatment as antimicrobial resistance to available drugs which is increasing day by day. However, it should be standardized and tested in animal models before its application.

Keywords: Disk diffusion method, alternative source, treatment, antimicrobial resistance, standardized

1. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative ubiquitous bacterium that can be isolated from sources such as soil, plants, animals and humans [1]. It is an important pathogen which causes various infections: Urinary Tract Infection (UTI), Respiratory Tract Infection (RTI), otitis media, skin and soft tissue infections, bacteremia and serious systemic infections particularly in people with compromised immune systems including burn sufferers, cystic fibrosis, cancer and AIDS [2, 3]. The increasing use of antibiotics and growing numbers of invasive procedures, together with the development of intrinsic and acquired resistance mechanisms of *P. aeruginosa*, cause the evolution of numerous multi drug resistant (MDR) *P. aeruginosa* outbreaks in clinical settings [4]. Although Carbapenems are the antibiotics of choice for several Pseudomonal infection, resistance is evolving due to production of MBLs which are broad spectrum enzymes that hydrolyses most beta-lactam antibiotics except Monobactams [5]. *P. aeruginosa* can form biofilms which exponentially increase antibiotic resistance [6]. Biofilms are the aggregation of cells which protect bacteria from environmental stresses as well as from the host immune system and antimicrobials [7]. The biofilms are composed of one or more extracellular polymers such as polysaccharide that holds the cell community together [8]. Metallo-**β**-lactamases are a diverse set of enzymes that catalyze the hydrolysis of a broad range of **β**-lactam drugs

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including carbapenems. The dissemination of the genes encoding these enzymes among Gram-negative bacteria has made them an important cause of resistance.

Synthetic antibiotics are widely used to cure infections; however indiscriminate use of such antibiotics causes antimicrobial drug resistance, necessitating the use of medicinal plants as the alternative therapeutic agents [9]. *Psidium guajava* L leave are important and commonly used to treat diseases like diabetes mellitus II, gastroenteritis, blood coagulation, gastric mucosal injury, etc. *P. guajava* leave contain active chemical compounds such as saponins, flavonoids, tannins, eugenol and triterpenoids. Poly phenolic compounds that dominate *P. guajava* leave are flavonoids (>1.4%) and tannins [10]. The activities possessed by *P. guajava* leave are antiviral, anti-inflammatory, anti-plaque and antimutagenic and thus, it can be helpful for prevention and treatment of diseases [11]. The main aim of this research is to find out the effect of *P. guajava* leave extract on multidrug resistant biofilm and metallo β lactamase producing pseudomonas respectively.

2. MATERIALS AND METHODS

2.1 Method

The method was quantitative carried out at the Microbiology laboratory of KIST Medical College and Teaching hospital, Gwarko, Lalitpur, a tertiary care hospital and Central Department of Microbiology, Tribhuvan University, Kirtipur, from April to October, 2018.

2.1.1 Specimen

Samples included pus, wound swab, blood, sputum and urine from in and out patients visiting the hospital.

1.1.2 Identification and Antibiotic Susceptibility Testing of Pseudomonas aeruginosa

The identification of *P. aeruginosa* isolates were carried out on the basis of standard microbiological procedures and antibiotic susceptibility testing was done by modified Kirby Bauer disc diffusion method following CLSI guidelines, 2014.

2.2 Detection of Metallo β Lactamase (MBL)

2.2.1 Screening of MBL Production by Imipenem Disk Diffusion Method

According to the CLSI recommendation, MBL production was screened using Imipenem disk same as antibiotic susceptibility test on MHA agar plate and resistant zone was noted. Resistant zone indicated a probable MBL producing strain which was further confirmed by phenotypic confirmatory test.

2.2.2. Confirmation of MBL production by Imipenem- EDTA Combined Disk Method

EDTA of 0.5M was prepared with distilled water and sterilized by autoclaving. Imipenem disk was supplemented with EDTA by dispensing 10µl of the solution to each Imipenem disk.

Imipenem-EDTA combined disk method (CDT) was performed. A lawn culture of test isolates was prepared. After allowing it to dry for five minutes, two Imipenem discs, one with 0.5 M EDTA and the other a plain Imipenem disc, was placed on the surface of agar plates approximately 30mm apart. The plates were incubated overnight at 37. An increase in zone diameter of ≥7mm around Imipenem + EDTA disk in comparison to Imipenem disk alone indicated production of MBL [12].

2.3 Detection of biofilm production

Biofilm detection by microtitre plate culture method (MPC):

The overnight grown cultures of *P. aeruginosa* from agar plates were inoculated in trypticase soy broth (TSB) with 1% glucose. Stationary-phase 18-hr culture of *P. aeruginosa* was diluted 1:100 with fresh TSB. Individual well of sterile polystyrene 96 wells flat bottom tissue culture plates were filled with 200µl of diluted culture broth. Uninoculated broth was considered as negative control. The plates were incubated at 37˚C for overnight. After 24 hours of incubation, content of each well was gently discarded by tapping the plates downwards. The wells were washed three times with 200µl of PBS (pH 7.2) in order to remove planktonic bacteria. Adherent bacteria were fixed with 99% methanol for 10-15 min. After drying the plates, stained for 10 min with 0.1% crystal violet (CV). Excess stain

was removed by washing the wells with distilled water and plate was kept for drying at an inverted position. After the plate was air dried, the dye bound to the adherent cells was re-solubilized with 160 µl of 95% ethanol. The OD of each well was measured at 570 nm using ELISA reader. These OD values were taken as index of bacteria that adhere to the surface and formed biofilm. Experiments were carried in triplicate and their mean was taken for the analysis. Interpretation of biofilm production was done according to the standard criteria [13].

2.4 Preparation of methanol and hydro ethanol extracts of *P. guajava* leave

Methanol extract was prepared by taking absolute methanol as solvent, hydro ethanol extracts by taking ethanol and water in different ratios (7:3, 1:1 and 3:7). The leave pieces were added to different solvents in sterile flasks and wrapped with aluminum foil to avoid evaporation. The mixtures were kept for 3 to 4 days at room temperature. The flasks were placed on a platform shaker at 70 rpm. After 3-4 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 (Figures 3, 4).

2.5 Determination of antimicrobial activity of *P. guajava* leave extractions against *P. aeruginosa*

Antimicrobial activity of *P. guajava* leave extract was tested by well-diffusion method according to the standard of the National Committee for Clinical Laboratory Standards. Diameter of 5 mm wells were punched into the MHA medium using a sterile cork borer and inoculated with the test bacterium. Exactly 0.1ml aliquots of each test extract were dispensed into each well. Methanol and hydroethanol solvents alone were used as control. Disk of Tobramycin was placed at the centre. After 24 hours of incubation at 37°C, each plate was examined for inhibition zones (mm).

2.6 Data analysis

The data obtained were analyzed by using SPSS software for Windows (version 21). A value of $\alpha \leq 0.05$ was assumed wherever applicable and 95% confidence intervals along with the exact p-values were presented.

Fig. 3. Antimicrobial activity of fresh *P. guajava* leave extract

MFLE: methanol fresh leave extract, EFLE1: hydroethanol fresh leave extract (5:5), EFLE2: hydroethanol fresh leave extract (3:7), EFLE3: hydroethanol fresh leave extract (7:3) and NS: normal saline

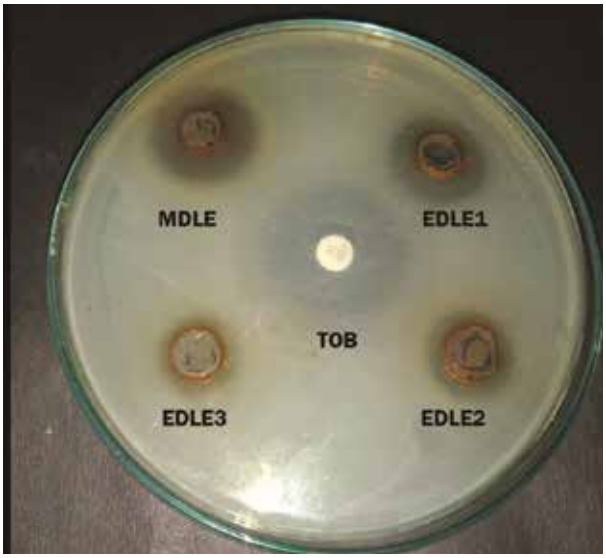

Fig. 4. Antimicrobial activity of dried *P. guajava* leave extract

MDLE: methanol dried leave extract, EDLE1: hydroethanol dried leave extract (5:5), EDLE2: hydroethanol dried leave extract (3:7), EDLE3: hydroethanol dried leave extract (7:3) and TOB: Tobramycin

3 RESULTS

A total of 3500 specimens were processed during the six months of duration. Bacterial growth was observed in 900 (25.71%) samples. Out of the total specimens, *P. aeruginosa* isolates were 45 (5%).

3.1 MBL and biofilm production in MDR *P. aeruginosa*

Out of 45 isolates of *P. aeruginosa,* 30 (66.67%) were MDR isolates. The total MBL producing *P. aeruginosa* was found to be 6 (13.33%) by Imipenem-EDTA combined disk method and all of them were found to be MDR strains while 24 (80%) were negative for MBL production. Out of 30 MDR isolates, 29 (97%) were biofilm producers of which 15 (50%) were strong and 14 (47%) were moderate biofilm producers and out of 15 non MDR isolates only 1 (7%) was found to be biofilm producer (Figure 1).

3.2 Antimicrobial activity of *P. guajava* leave

P. guajava leave extract (fresh and dried) showed antimicrobial activity against all types of *P. aeruginosa* isolate, both drug resistant and drug sensitive. However, the activity was quite lower than Tobramycin (16mm), the antibiotic standard used, in both ATCC culture and clinical culture of *P. aeruginosa.* Methanol extract of fresh leave extract showed higher activity (13mm) than that of dried leave extract (12mm). Among different concentration hydroethanol extract, 5:5 hydroethanol extract showed greater inhibition followed by 3:7 hydroethanol extract and least by 7:3 hydroethanol extract. However, the activity was comparatively high in fresh extracts than in dried extracts (Figure 2).

4 DISCUSSION

Resistance to antimicrobial agents is an increasing clinical problem and is a recognized public health threat. *P. aeruginosa* showed a particular propensity for the development of resistance. The emergence of resistance in *P. aeruginosa* also limits future therapeutic choices and is associated with increased rate of mortality and morbidity [14, 15]. This study was carried out to assess MDR, MBL production, biofilm production and antimicrobial activity of *P. guajava* leave extract in *P. aeruginosa* isolated from different clinical specimens of in patients and out patients at Medical College and Teaching Hospital. The total of 45 (5%) isolates of *P. aeruginosa* were isolated and followed by antibiotic susceptibility testing. MDR was shown by 30 (66.67%) of *P. aeruginosa* isolates tested. Similar study made by Fatima et al. (1999) showed 73.9% MDRPA isolates [16]. Drug resistance in *P. aeruginosa* is multifactorial either through membrane permeability and efflux system or through its virulence factors or acquired genetically by plasmid which may lead to a super bugs, that are difficult to treat [17]. Emergence of MDR is related

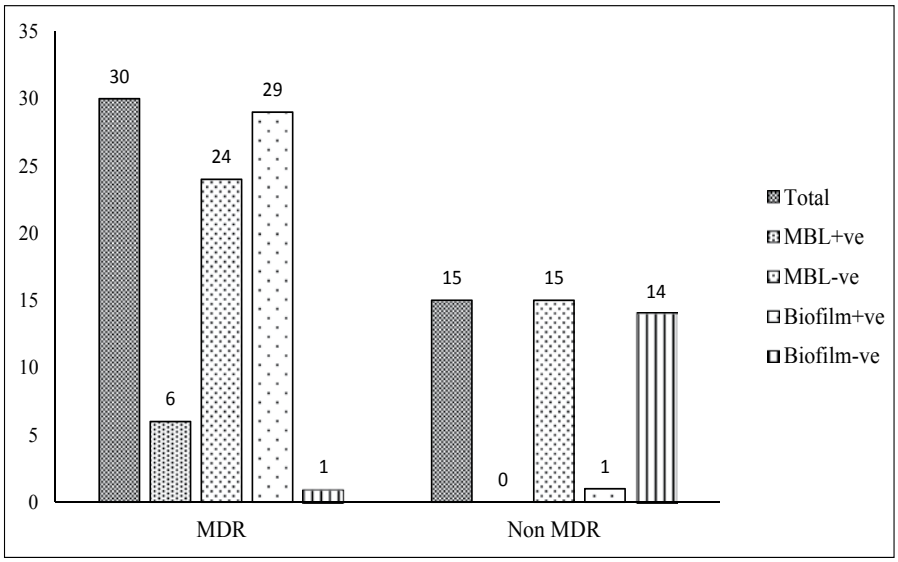

Fig. 1. MBL and biofilm production in MDR and non MDR *P. aeruginosa*

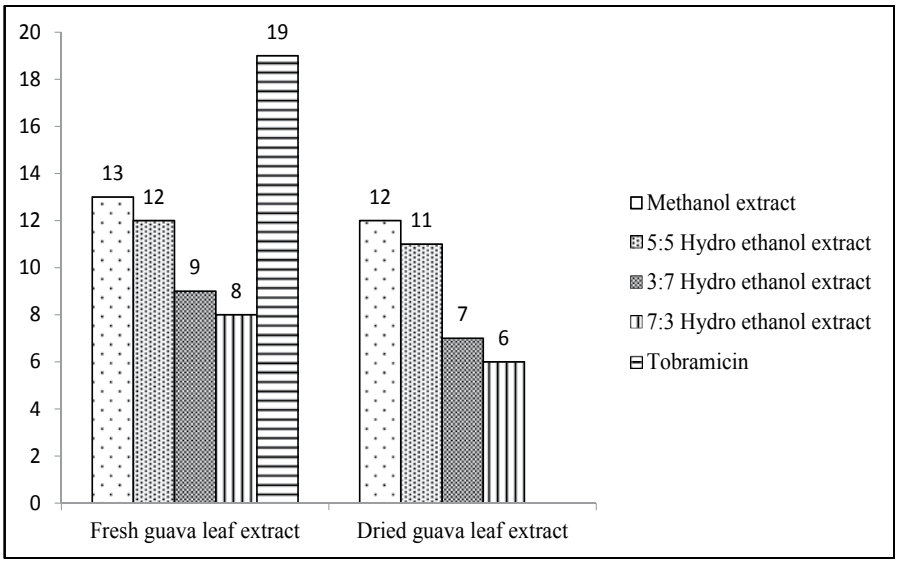

Fig. 2. Antimicrobial activity of *P. guajava* leave extract on *P. aeruginosa* isolates

to the empirical use of antibiotics rather than their rational use as majority of patients undergo broad spectrum antibiotics before sample collection. Drug resistance should be prevented by encouraging judicial use of antibiotics.

In this study, out of 45 isolates, 6 (13.33%) *P. aeruginosa* isolates were found to be positive for MBL production with MDR. Similarly, Thapa et al (2017) reported 14.2% of MBL producing *P. aeruginosa* isolates during the study [18]. Our result varied much more compared to Acharya et al (2017) who reported 68.6% positive isolates for MBL production by the Imipenem-EDTA disk diffusion test [19]. MDRPA has appeared as an issue of great concern with emergence of MBLPA [20]. Carbapenem resistance in *P. aeruginosa* is most commonly due to production of MBL [21]. Carbapenem resistant *P. aeruginosa* has become prevalent globally [22, 23]. MBL production in *P. aeruginosa* is associated with treatment failure, longer hospital stay and significant morbidity and mortality [24]. Routine detection of MBL may ensure optimal patient care and timely introduction of appropriate infection control procedures [25].

In this study, 30 (66.67%) biofilm producing *P. aeruginosa* were isolated. Out of total biofilm producer, 29 were MDR and their association was statistically significant (<0.05). Similar study made by Maita et al (2014) showed biofilm producers statistically significant while all non-biofilm producers were non MDR. The *P. aeruginosa* isolates producing biofilm were reported high about 79.4% [27]. Antimicrobial resistance is an innate feature of bacterial biofilms and many studies have shown that biofilm formation is higher in MDR strains [28]. One of the most medical important biofilm forming bacteria is *P. aeruginosa* which is usually associated with human nosocomial infections [29, 30 and 31]. Extracellular matrix of biofilm acts as barrier for any antibiotics and increase resistance to these antibiotics [8]. Biofilm producing bacteria are 10 to 1,000 times more resistant to antimicrobial agents than the planktonic cell. There is high level of antibiotic resistance among biofilm-forming *P. aeruginosa* strains. The differences in the various reports about the prevalence of biofilm formation may be attributed to the variation in the sites of infection, multiple subcultures of bacteria, method of biofilm detection, species-specific and bacterial strain [32]. Regular screening of biofilm formation and monitoring antimicrobial resistance profile of *P. aeruginosa* is very important as it may help to formulate an effective antimicrobial strategy in a clinical setting while dealing with infections caused by this organism [26].

In the study, *P. guajava* leave extract (fresh and dried) showed antimicrobial activity against all types of *P. aeruginosa* isolates both drug resistant and drug sensitive. However, the activity was quite lower than the antibiotic standard used Tobramycin (16mm), in both ATCC culture and

clinical culture of *P. aeruginosa.* Methanol extract of fresh leave extract showed higher activity (13mm) than that of dried leave extract (12mm). Among different concentration hydro ethanol extract, 5:5 hydroethanol extract showed greater inhibition followed by 3:7 hydroethanol extract and 7:3 hydroethanol extract respectively. However, the activity was comparatively high in fresh extracts than in dried extracts. Similar study made by Gitika et al (2016) reported maximum zone of inhibition for the methanol extracts of *P. guajava* leave [33]. The antibacterial activity of different *P. guajava* extract was found to be significant against both *E. coli* and *P. aeruginosa* but less significant than the standard antibiotics [34]. In another study, the methanol and ethyl acetate extracts were found to exhibit broader spectrum activity and the methanol extract was more active comparatively [35]. Ethanol was reported as the best solvent compared to acetone for tannin extraction from *P. guava* leave [36]. Khadka 2018 made a similar study where 83.67% isolates of *P. aeruginosa* were biofilm producer and *P. guajava* leave tea was able to kill *P. aeruginosa* [37]. It was analyzed that higher levels of tannin content was equivalent to higher antibacterial activity. The leave contain many fungistatic and bacteriostatic agents and important oxidants [11]. Synthetic antibiotics are widely used to cure infections, but necessitating the use of medicinal plants as the alternative therapeutic agents. Medicinal plants and plant-derived products are cost-effective and easily obtainable and have promising efficacy to treat infectious diseases, and thus they may be useful in eradicating new emerging microbial strains [9]. Recently, scientists have found evidence that specific combinations of phytochemicals are more effective in protecting against diseases than the isolated compounds, pointing to a need to study the synergy among active compounds in plants, for example, by experimenting with plant extracts [38].

5 CONCLUSION

Multidrug resistance *P. aeruginosa* create great challenges in the therapy. Effective means need to be developed to control this problem. Regular antimicrobial susceptibility surveillance may assist in monitoring of the resistance patterns antibiotic policy and introduction of effective antibiotics for better patient management. Biofilm production and MBL production in *P. aeruginosa* was found to be linked with MDR property of the organisms. Thus, early detection of biofilm production and MBL production may be helpful in controlling the infection by resistant strains. Many researches have been demonstrating the presence of a wide variety of bioactive compounds in *P. guajava* leave capable of showing beneficial effects on human health. In the present study, *P. guajava* leave extracts with methanol and various concentrations of ethanol showed significant inhibitory activity against *P. aeruginosa* isolates with fresh leave showing more activity than dried one. On the basis of this study, it can be said that *P. guajava* leave extracts can be effective antibacterial agent that can be a good source to treat and control many diseases. However, extensive research in clinical trials needed so that it can be used for prevention.

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