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Original Research Article

Effect of Piper Cubeba Extract on Biofim Producing Pseudomonas Aeruginosa Isolated from Clinical Samples

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Abstract

Background: The species of *Pseudomonas aeruginosa* is a nosocomial bacterium that increases the risk of multidrug-resistant infections, especially in vulnerable patients. The plant *Piper cubeba*, commonly referred to as cubeb or tailed pepper, it is specifically cultivated for its fruit and essential oil. The plant belongs to the genus *Piper*. The study aims at assessing the effectiveness of *Piper cubeba* extract against different *P. aeruginosa* strains that could produce biofilm and carry blaIMP gene. ***Method:*** Isolates from 85 samples (17 sputum, 40 urine and 28 diarrhea samples). Collected from Al Nasiriyah Teaching Hospital during the period of December 2021 to March 2022. Samples were cultivated using routine cultivation methods, catalase test, oxidase reaction, and the Api20E system were performed to identify *P. aeruginosa.* ***Results:*** Out of a total of 17 isolated samples of *Pseudomonas aeruginosa*, 3 (17.64%) were from sputum, 6(35.29%) from urine, and 8 (47.05%) diarrhea samples. Out of 17 isolates 6 were sensitive in stool isolates 4 isolates were moderate sensitive and 7 isolates were resistance. Conclusion at concentrations of 25, 50, 75, and 100 mg/mL for *Piper cubeba*, respectively, the greatest MBC of plant extracts was noted. Also, all the isolates were capable of producing a biofilm by using presterilized 96-well polystyrene microtiter plates. Out of 17 isolates, 9 had a potent biofilm ability and the remaining 7 were moderate biofilm producers. Additionally, 7 of the isolates (41.17%) carried the blaIMP gene. ***Conclusion:*** *Piper cubeba* extract has potential as an antimicrobial agent against both sensitive and moderately resistant strains of *Pseudomonas aeruginosa*. This antimicrobial effect becomes more pronounced with an increase in the concentration of the extract. Additionally, *Pseudomonas aeruginosa* that produce biofilms tend to consistently produce more Metallo-beta-lactamase (MBL), which can further contribute to antibiotic resistance.

Keywords: *Piper cubeba*, *Pseudomonas aeruginosa*, biofilm, MBL.

INTRODUCTION

antibiotic overuse is increasingly linked to bacterial resistance in both commensal and harmful microorganisms that serve as a source of resistant determinants (H. V. Nguyen *et al*., 2016). Essential oils and secondary metabolite chemicals from medicinal plants are abundant sources of pharmaceuticals that are safe for competing infections. Furthermore, combining antimicrobial medications can result in beneficial interactions against microorganisms. This can lessen the negative effects of antibiotics, increase bio-availability, and decrease the therapeutic dose. (Q. H. Nguyen *et al*., 2019),(Yap, P.S.*et al*., 2014). The primary inhibitor of the phagocytosis cycle is the size of the bacterial biofilm. In fact, portions of the resistant architecture are only seldom viable against biofilm contaminations in immunocompetent individuals. Following immediate interaction with tiny organisms, neutrophils and macrophages swiftly activate as part of the inborn invulnerable reaction. (Olivares, E. *et al*., 2020). *Piper cubeba* is a significant species that is used in traditional medicine to treat rheumatism, chills, the flu, colds, muscular aches, and fever. Biofilm-bound bacteria can withstand antimicrobial agents up to ten folds better than their plank tonic counterparts and avoid insensitive reactions. *P. aeruginosa* is a famous biofilm producer, thus serving as an excellent model for biofilm development research. *P. aeruginosa* requires a strong biofilm to survive, thrive, and govern in the polymicrobial environment of the cystic fibrosis lung. (Thi, M.T.T. *et al*., 2020). The *P. cubeba* essential extract's chemical composition and potential inhibitory activity against pathogenic bacteria enzyme. Gas chromatography and mass spectrometry (GC-MS) showed that the primary components were

methyleugenol and eugenol. Using phytochemical analysis of the DCM fraction, asarone and asaronaldehyde were successfully extracted and classified from the methanol extract and its fractions of *P. cubeba*.(Alqadeeri *et al*., 2019). There is currently no recognized method for preventing or eliminating the growth of undesirable biofilms without causing negative side effects. The increase of biofilm resistance to traditional medications has disrupted the need to seek for novel control strategies. The hunt for safer, greener alternatives to anti-infection drugs and chemical additives is unending, particularly with the development of phyto-medicine on the horizon. Plant-derived antimicrobials are widely recognized due to their long history of use and perceived health benefits. (Onsare, J. G., & Arora, D. S. 2015).

MATERIAL AND METHODS

Characterization of Bacterial Isolates:

Sputum and urinary tract infection samples were collected and inoculated on blood and MacConkey agar plates for bacterial isolation before being incubated at 37°Celsius for 24 hours. The individual colonies were identified by traditional microbiological techniques (Ansar and Dastagir, 2018). Traditional methods such as colony morphology, pigment production on selective media, the catalase test, an oxidase reaction, and the Api20E system were employed to identify the isolates. (Shehab, and Jassim, 2019).

Preparation of Crude Extract:

Hot water was used to make *P. cubeba's* fruit extract. With the aid of a magnetic stirrer, 30g of fruit is dissolved in 100ml of hot (100 CE%) water, then allowed to stand for five hours. after which filtered with sterilized Whatman filter paper. Filtered extracts were preserved in sterile containers with labels in a deep freezer at -18°C after being air dried at 40°C for 48 hours. (Al-daan *et al*., 2017).

Agar Well Diffusion Method:

The Okeke agar-well diffusion technique has been used to test extract's antimicrobial activity. In this procedure, Mueller Hinton Agar-containing media plates were used to cultivate pure isolates of microorganisms for 24 hours at 37°Celsius. In the inoculated plates, 100 microliters of inoculum of each test organism were dispersed in 6 mm-diameter wells using a sterile borer. Specifically inoculated medium agar plates (Mueller Hinton Agar) had 100 µl of extract injected into each well. The inoculated plates, then allowed to stand for about 10 minutes to permit the extract to diffuse before being incubated at 37°Celsius for 24 hours. The -ve controls were sterile distilled water and 20% DMSO, whereas the +ve control was 0.2% Ciprofloxacin (Jain *et al*., 2015).

Biofilm Formation Assay:

By using the 96-well microtiter plate wells' capacity to hold cells in place After incubating. The 96-well polystyrene microtiter plates for 48 hours at 37 °C with 200 microliters of this bacterial culture, all wells were rinsed with sterile physiological saline to remove any unattached cells. Then, each well received 200 µl of 1% crystal violet. After 15 minutes at room temperature, each well was cleansed with 200 µl of sterile physiological saline. This method was repeated three times. The absorbance at 540 nm was measured using an ELISA reader after extracting the crystal violet from the biofilm with 200 µl of ethyl alcohol. (Kadhum, and Khudor, 2021).

Molecular Methods

Genetic evaluation for the production of MBL was carried out following directions provided by the manufacture's manual for the Genomic DNA Mini-Kit (Geneaid, New Taipei City Taiwan), DNA was extracted. The blaIMP gene was detected by PCR using the previously published primers. The final volume of the PCR reaction was 20 µl, comprising 12.5 µl master-mix, 5µl DNA-template, and 0.5 µl primers (Kapa, Cape Town, South Africa). A thermo-cycler instrument (A&B Singapore) has been used to measure the reaction conditions. (Poirel *et al*., 2011). For the coding portions of the genes blaIMP-F (5"GGAATAGAGTGGCTTAAYTCTC"3) and blaIMP-R (5"GGTTTAAYAAAACAACCACC"3) (232 bp), specific primer sequences were used in PCR experiments. The reaction volume for blaIMP gene was 20µl, and the amplification schedule included three cycles at 95 degrees Celsius for denaturation, 30 cycles each for annealing at 60 degrees Celsius for 45 seconds, extension at 72 degrees Celsius for 50 seconds, and final extension at 72 degrees Celsius for 10 minutes(Alkhudhairy, and Al-Shammari, 2020).

RESULT

Of 85 sputum, urine, and diarrhea specimens were tested, and 17 (20%) *P. aeruginosa* were found, with 3 (17.6%) of those coming from sputum, 6 (35.2%) from urine, and 8 (47.0%) from diarrhea samples. The findings revealed that diarrhea patients had a higher prevalence than those who had urine samples.

Six of the 17 isolates tested had sensitivity. The majority of sensitive isolates were found in stool isolates, of which four were somewhat sensitive. While there were 7 resistant isolates, there were only 1 sensitive isolate (urine) and 5 diarrheal isolates. While some isolates were moderately sensitive (1 sputum, 1 urine, and 2 diarrhea) while others were resistant (4 urine, 2 sputum, and 1 diarrhea). Extract from plant were showed to be more effective, resulting in wider zones of growth suppression. Bacterial activity affected with concentration of higher doses (100 mg/ml) (table 1) (Fig 1). Also, all *P. aerogenosa* isolates were able to produce

biofilm (100%). by using presterilized 96-well polystyrene microtiter plates (Fig 2) Nine *P. aeruginosa* isolates produced strong biofilms, whereas seven produced intermediate biofilms. Seven isolates. (41.17%) harbored the blaIMP gene and produced MBL (Table 2). (Fig 3).

Table (1): Effect Antibacterial of alcoholic piper cubeba extract against *Pseudomonas aerogenosa*

Pseudomonas aerogenosa	**Concentration mg/ml / *Piper cubeba* Extract**			
	25 mg/ml	**50 mg/ml**	**75 mg/ml**	**100 mg/ml**
NO. sensitive\ Zone of inhibition mm	-	-	2 \ 8 mm	4 \ 11mm
NO. moderate sensitive\ Zone of inhibition mm	-	1 \ 3mm	1\ 6mm	2\ 10mm
NO. resistance	3\0	2\0	1\0	1\0
control\ Zone of inhibition (mm)	Ciprofloxacin \ 12mm (Positive control)		20% DMSO\ 0 (negative control)	

Table (2): Biofilm formation ability of isolates by*Pseudomonas aeruginosa* microtiter plate method

Sample	**NO. of isolate**	**Biofilm prouder**		
		Weak No.	**Moderate NO.**	**Strong NO.**
Sputum	3	0	3\17.64	0
Urine	6	0	2 \ 11.76	4 \ 23.52
Diarrhea	8	1\ 5.88	2\ 11.76	5\ 29.41
Total	17	1 \ 5.88	7\ 41.17	9\ 52.94

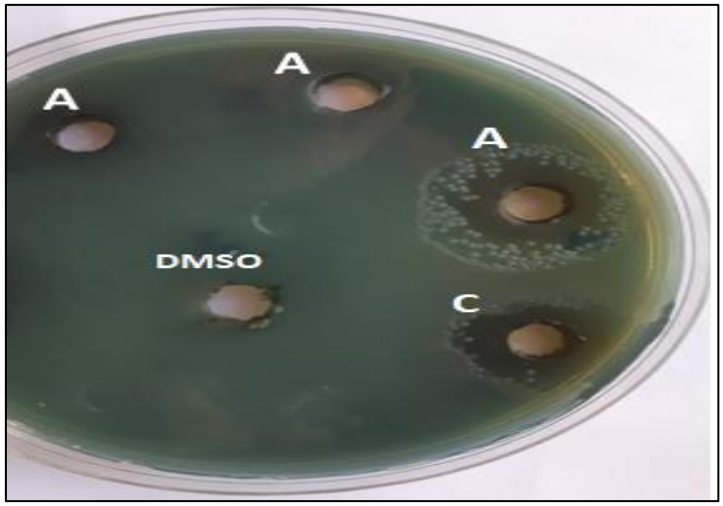

Figure (1) A: Antibacterial of *piper cubeba* extract against *pseudomonas aerogenosa* C: Ciprofloxacin (Positive control) and 20% DMSO (negative control)

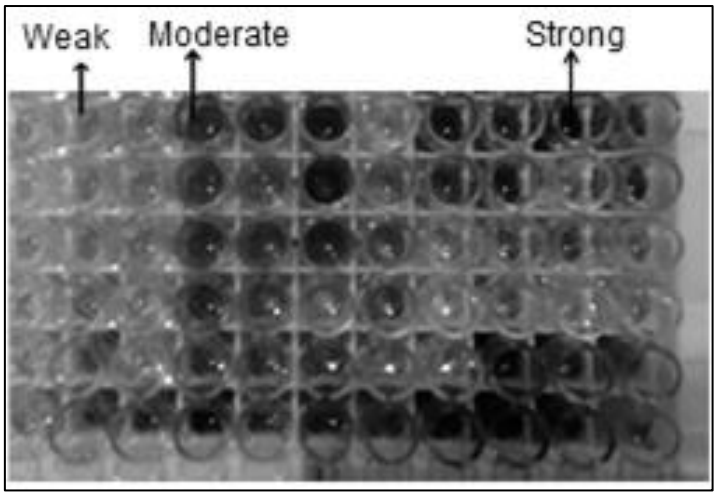

Figure (2): 96-well polystyrene microtiter plates for biofilm formation in tested isolates

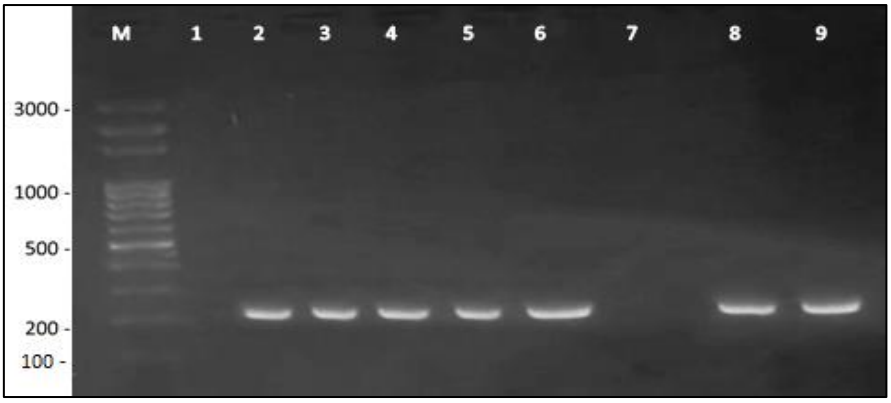

Figure (3): *Pseudomonas aeruginosa* blaIMP gene gel electrophoresis using standard PCR.

DISCUSSION

The rise of antibiotic-resistant bacteria is a major danger to world health, necessitating the search for alternate sources of effective antimicrobial medicines. This study examined the effectiveness of *Piper cubeba* extract against biofilm-producing *Pseudomonas aeruginosa*, a bacterium known for drug resistance. Plants have a significant role in maintaining people's health and enhancing their quality of life. Herbal plants contain anti-inflammatory properties (Alborzi, A., B. A 2000).

The study looked at the concentration-dependent inhibitory impact of *Piper cubeba* extract on *P. aeruginosa*. The data revealed a dose-dependent response, with higher concentrations (100 mg/ml) being more beneficial. The extract's impact was assessed using zone of inhibition experiments, which yielded promising results. Concentrations of 100 mg/ml produced the broadest zones of growth suppression, indicating that *Piper cubeba* extract may have a role in fighting *P. aeruginosa* infections, the same effect was observed using different species of Piper, namely *Piper betle* by M.F. Siddiqui *et al.*, suggesting that *Piper* extract had a bacteriostatic effect on *P. aeruginosa*, which could be caused by the *Piper* extract interfering with bacterial environmental factors and produced a non-favorable conditions which limited their growth and resulted in increase inhibitory zone. These finding are consistent with previous study that reported the effect the *Piper* extract could be the results of it interfering with quorum sensing by Irene Ratridewi *et al.*,

P.aeruginosa was the most common causative organism of, 6(35.2%) from urine, 8 (47.0%) diarrhea samples. However, The current results are consistent with these of Bhat *et al*., 2021. All agar-based procedures, including diffusion methods, need a lot of work and time. Based on the higher levels that the diffusion method revealed, plant extracts have antibacterial action at lower doses than the disk diffusion approach. The agar dilution approach is better suited for determining antibacterial activity quantitatively. (Klančnik *et al*., 2010). Plant extracts demonstrated good antibacterial action against *P. aeruginosa*, which is consistent with prior findings of Akshita *et al*., (2020) and also with Ahmed and Hameed (2017). That assessed for the production of biofilms using phenotypic screening. The micro-titer plate's applicability, dependability, and high reproducibility were previously verified for bacterial biofilm, and during their investigation, Abdulhaq *et al*., (2020) discovered a high level of agreement for isolate phenotypes created using these plates. The occurrence of MBL was reported to be 41% in the current investigation (7 out of 17 *P. aeruginosa* isolates). This rate was comparable to that found in other research like Dogonchi *et al.*, 2018. Prevalence of MBL producers 40 % in northern Iran. Various percentages of MBL producing *P. aeruginosa* have been recorded in different regions, such as 38.3% in São Luis, Brazil, 47.3% in Taiwan, and 62% in some regions of Greece Akya *et al*., (2015), Saderi, and Owlia (2015).

CONCLUSION

Piper cubeba extract has potential as an antimicrobial agent against both sensitive and moderately resistant strains of *Pseudomonas aeruginosa*. This antimicrobial effect becomes more pronounced with an increase in the concentration of the extract. Additionally, *Pseudomonas aeruginosa* that produce biofilms tend to consistently produce more Metallo-beta-lactamase (MBL), which can further contribute to antibiotic resistance. More research is needed to identify pharmacologically active components using bioactivity-guided fractionation.

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Conflict of Interests: There are no conflicts of interest to disclose.

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