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Effect of Essential Oil *Cinnnamomum Zylanicum* on Biofilm Producing MDR Clinical Isolates of *Pseudomonas Aeruginosa*

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***Abstract*— *Pseudomonas aeruginosa* is an opportunistic pathogenic bacterium associated with various infections. Along with its resistance to drugs, the biofilm forming strategy has drastically reduced effectiveness of antipseuodomal drugs as well as reduced provoking appropriate immune response. Biofilm are extracellular polymeric substances (EPS) that hold microbial cells together to a surface. This present condition focuses on remedial source to treat this very notorious multi drug resistant (MDR) biofilm producing strain of *P. aeruginosa*. Quorum sensing (QS) is chemical signalling mechanism controlling virulence factor with biofilm formation ability. Biofilm formed by the population of bacteria act as a protective environment. Bacteria sustaining in biofilms demonstrate distinct features like different physiology and immense resistance to immune system and antibiotics, biofilm act a source of chronic and persistent infections. Various diseases are associated with the biofilm forming property of ubiquitous microorganism. It is essential to implement a better parameter for antibiotics resistant strains. Solving this problem present study aims at biofilm inhibitory activity of *Cinnnamomum zylanicum*.As biofilm act as major factor for drug resistance, essential oils potentially inhibit biofilm producing MDR strains. The *Cinnnamomum zylanicum* oil had strong inhibitory activity against multidrug resistant strains and its biofilm inhibiting activity contribute over it.**

Index Terms* — Antibiotics, Biofilm, Cinnamon oil, Essential oil, MDR, *Pseudomonas aeruginosa

I. INTRODUCTION

P. aeruginosa a ubiquitous bacterium has emerged as a dreaded pathogen in term of causing multidrug resistance and persistence infection. Among opportunistic pathogenic bacteria *P. aeruginosa* produces several virulence factors, is known to be an important human and plant pathogen, responsible for various infections, particularly in immune compromised person [1]. Besides this, the remarkable ability of *P. aeruginosa* to form biofilms in many environments renders antibiotic treatments inefficient and therefore promotes chronic infectious diseases [2], [3].The ability of micro-organisms to develop biofilms makes them even harder to eradicate as these structures are more resistant than the planktonic forms [24]. Bacteria within biofilms are protected from antibiotics and disinfectants than individual cells in suspension [16].Biofilm formation represent a protective mode of growth, helps microorganisms to sustain in hostile environments and dispersing cells to colonize new positions under favourable conditions. Once biofilms develop into a mature stage, they become extremely difficult to eradicate from infections sites with traditional antimicrobial agents [4]. Agents that inhibit biofilm formation or transform bacteria from biofilm life style to free-living individuals are ideal to eradicate biofilm [5]. Essential oils and their components are widely used in medicine as constituents of different medical products, in the food industry as flavouring additives and also in cosmetics as fragrances [6]. Furthermore, many Essential oils are relatively easy to obtain, have low mammalian toxicity and degrade quickly in water and soil, so they are relatively environmentally friendly[7].

Essential oils and their components resulted to be good inhibitors of biofilm formation [8] or even better than some antibiotics against these structures [9]. Cinnamon is a dietary phytochemical that shows antimicrobial properties and is particularly significant given that dietary chemicals are deemed trustworthy and used habitually in daily life. Cinnamon is conventionally endorsed for treating digestive problems, including nausea, vomiting, and diarrhea [10]. The wrong and excessive dose of antibiotics is a serious problem in antimicrobial chemotherapy which causes resistance and ineffective antimicrobial treatment [11]. This, antibiofilm compounds could be interesting antibiotic adjuvants to prevent or treat chronic infections [12]. The objective of this study was to evaluate the antibiofilm activity of cinnamon oil on MDR clinical isolates of *P.aeruginosa.*

II. MATERIALS AND METHODS

A. Medicinal Plant:

Cinnnamomum zylanicum (Dalchini)

B. Extraction Procedure:

The bark of above mentioned plants were dried up at room temperature. After cleaning and removal of the sand and foreign material, all the dried material was ground to fine powder using a grinder. The oil was extracted with n-hexane (1:4 w/v) by continuous extraction in a soxhlet apparatus for 12 hours.

C. Microbial strains:

A total of 12 clinical isolates were isolated from patients and were procured from department of microbiology (Pravara Rural Hospiatal), Pravara instituite of Medical Science-DU,Loni. Sample Processing and identification of organism was done in microbiology department: Two sterile swab sticks were used to collect the pus samples.

1st swab stick was used for gram staining and IInd swab

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stick was used for culture. Direct smear with gram stain were screened for the presence of inflammatory cells and type of microbial flora. II[nd]swab was inoculated on MacConkey agar (MA) then it was incubated at 37°C for 24 - 48 hrs. After observing the growth on MA, it was then subcultured on MA &. The colonial morphology and identification was done as per standard microbiology procedures [13].

D.Antibiogram Testing:

Selective colonies from the culture plate were inoculated into 2ml of peptone water. Incubated at 37^0 C for 2 hr. Turbidity was compared to that of 0.5 McFarland standards. A cotton swab was immersed and rotated in this inoculums, the swab was then pressed to the inner surface of the tube so as to remove excess inoculums. It was then used for carpet streaking on Muller Hinton agar plate. The required antibiotic discs were then placed aseptically on this medium with sterile forcep.

The plate was then incubated 24 hr at 37^0 C. Next day the zone size was recorded and reported as sensitive or resistant by comparing the zone size to the Kirby-bauer chart. Antimicrobial susceptibility testing of isolates was performed by standard Kirby Bauer disc diffusion methods according to CLSI protocol [14].Depending on the isolate that is *Pseudomonas aeruginosa*, antibiotic discs were selected from among the following to determine antibiotic sensitivity pattern of *Pseudomonas aeruginosa* isolates:

Gentamicin (GEN) (10mcg/disc), Meropenem (MRP) (10 mcg/disc), Cefazolin (CZ) (30 mcg/disc), co-trimoxazole (COT) (25 mcg/disc) was tested (HIMEDIA, MUMBAI, INDIA).

Similarly antimicrobial activities of the essential oils were studied with the well diffusion method. Wells 5 mm in diameter were punched into the agar and filled with 20–30 of the oils. The plate was then incubated 24 hr at 37^0 C and the end of the period, the diameter of inhibition zones we measured in (mm) using a vernier scale.

E. Evaluation of biofilm forming potential:

The biofilm forming potential of *P.aeruginosa* was evaluated by observing visible film lining the wall and the bottom of the tube along with a film at air - liquid interface acting as a protective environment for bacterial population. It can be more distinct when treated with 2 ml of crystal violet staining solution .A loopful of culture was inoculated in Luria burteni (LB) broth .The tubes were incubated at 37°C for 24 hrs. The intensity of biofilm formed was scored as 1- weak/none (0), 2- moderate (++) and 3- high/strong (+++) [18],[19].

The score calculation for tube method was done according to the results of the control strains. The experiment was performed in triplicate and repeated three times for all MDR strains of *P.aeruginosa.*

F. Biofilm Inhibition Assay

Tube test:

The study for the effect of cinnamon oil on biofilm formation was adapted from Christensen, et al. [17].A Plastic conical tubes/glass tube was inoculated with loopful fresh bacterial culture containing Luria burteni (LB) broth/Muller Hinton broth.Cinnamon oil/ Dalchini oil was added in variable concentration of 10%, 20%, 30% (v/v).The tubes were incubated at 37° C for 24 h. Tubes with inoculum only and with medium only were used as positive and negative controls.

Subsequently after incubation, air- liquid interface of the tube for biofilm integrity was observed the contents of each tube were carefully siphoned off and discarded. 2 ml of crystal violet solution (Merck, Darmstad, Germany) were added to each tube and left to react for 5 min on a roll and tilt mixer for providing homogeneous staining condition and the staining solution was discarded, the tubes in inverted position was left for drying overnight at room temperature. Biofilm formation was judged qualitatively by observation of a visible film lining the walls ,at bottom, and estimated as absent (0), weak (+), moderate (++) or strong (+++) [19]. All assays were done in triplicate.

III. RESULT

A total of 12 Multi drug resistance (MDR) isolates were selected for this study. MDR strain was detected for ≥ 3 antibiotics group.According to the antibiogram analysis in the following data (**Table I**) all the isolates were resistance and followed a unique pattern of resistance. Whereas cinnamon oil had much greater impact on this MDR isolates (**Table II**).

Table I: Antibiogram analysis of *Pseudomonas aeruginosa*

Sr.No.	Antibiotic Resistant Pattern	Total number of isolates	Isolates
1	CZ^r,MRP^r,COT^r,GEN^r	12	P1,P2, P3,P4, P5, P6, P7,P8,P9, P10,P11,P12

Out of 12 clinical MDR isolates were 100% biofilm producing. The biofilm producing potential was evaluated under the score of strong (+++) biofilm production [17]. Biofilm production was observed at the wall lining, bottom and at air – liquid interface (**Figure I**).

Figure I: Strong biofilm production by MDR clinical isolates of *P.aeruginosa.*

Table II: Effect of *Cinnnamomum zylanicum* oil on *Pseudomonas aeruginosa*

	Essential oil	MDR (Biofilm producing)											
r.No.	Medicinal Plant	Essential oil inhibitory effect on clinical isolates (mm)											
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
1	*Cinnnamomum zylanicum* (Dalchini oil)	24 (++++)	21 (++++)	26 (++++)	25 (++++)	28 (++++)	24 (++++)	28 (++++)	28 (++++)	25 (++++)	28 (++++)	23 (++++)	27 (++++)

* Diameter of inhibition zone: (++++) - 20mm and more; (+++) - 12-20 mm; (++) - 6-12 mm; (+) - 2-6 mm; (-) no antibacterial activity; 0 – not tested [15].

Table III: Inhibitory effect of *Cinnnamomum zylanicum* oil on *Pseudomonas aeruginosa.*

	Essential oil	MDR (Biofilm producing)											
Sr.No.	*Cinnnamomum zylanicum* (Dalchini oil)	Essential oils inhibitory effect on clinical isolates											
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
1	10 % (v/v)	+++	++	++	+++	+++	+++	++	+++	++	+++	++	++
2	20 % (v/v)	++	+	++	++	+	++	++	+	+	++	+	+`
3	30 % (v/v)	+	0	+	+	+	0	+	+	+	+	0	+

Biofilm Inhibition Assay (Tube test)

Cinnnamomum zylanicum oil is a boon to treat and in controlling various microbial strategies including biofilm formation. The efficacy of essential oil was evaluated by test tube method for biofilm inhibition (**Table III**).

This method was principally described by Christensen *et al*. [17], results procured by visual inspection are qualitative and more particular regarding cinnamon oil effect. In case of lack of film (0) negative control (medium without inoculum) was used for illustration and strong biofilm formation (+++) with positive control (bacteria without cinnamon oil). The levels weak (+) and moderate (++) were considered at intermediate levels [19]. The results ended up with cinnamon oil effect increased inhibition pattern from lowest to high concentration for all 12 MDR strains. Inhibitory effect was observed significantly.

As per the result **(Table III)** low concentration of cinnamon oil was significant, observed in some sample with moderate (++) biofilm for P2,P3,P7,P9,P11,P12 but increasing concentration is more effective .When the concentration is increased by 20 % (v/v) correspondingly add to inhibitory effect like in isolate P2,P5,P8,P9,P11,P12 were weak (+) biofilm observed. Further increased concentration of oil reduces integrity and viability of biofilm to a better extend revealing a clear pattern. There was no (0) biofilm observed in case of P2, P6, P11 were comparable with negative control. Thus increasing concentration of cinnamon

oil has an impetus on biofilm inhibition from strong to intermediate and no biofilm.

IV. DISCUSSION

As per the present scenario of various hospital settings, patient striving for life due to multi drug resistance (MDR) species sustaining in presence of various highly potent antibiotic therapy available . Rather than planktonic growth microbes evolved a strategy to survive in presence of a unfavourable environment provided by chemotherapy, by meansof biofilm forming potential – a source of chronic and persistent infections. *Pseudomonas aeruginosa* a notorious multi drug resistant and biofilm producing organism has demonstrated this scenario in such a way to hope for an better alternative to overcome challenges posed by it resistance and complete failure of drug therapy. Though Biofilms are difficult to eliminate with antibiotics there exist a natural and traditional alternative namely essential oil. Cinnamon oil a multipotent essential oil possessing antibacterial and biofilm inhibitory effects.

The present study conducted on 12 clinical isolates of rural tertiary care hospital of Pravara Institute of Medical Sciences,Loni. These isolates are classified as MDR as they are totally resistant to antibiotics and follow a unique resistance pattern CZ^r,MRP^r,COT^r,GEN^r against the antibiotics tested. These isolates followed similar resistance pattern like previous study [20]. All 12 isolates showed strong biofilm forming potential. These clinical isolates were then further tested for essential oils *Cinnnamomum zylanicum* (Dalchini oil). As reported in the previous study among various oil tested maximum activity was revealed for *Cinnnamomum zylanicum* (Dalchini oil), followed by *Eucalyptus globulus* (Nilgri oil), *Eugenia caryophyllata* (Clove oil), *Ocimum sanctum* (Tulsi oil) and *Allium sativum* (Garlic oil) .In-vitro efficacy of *Cinnnamomum zylanicum* was well executed in previous study, similarly recent study revealed a strong antibacterial property for MDR strains of *P.aeruginosa*. A significant zone of inhibition was achieved for MDR strains.

A report by Tarek *et al* stated that Cinnamon was the only essential oil which showed antibacterial activity against *P. aeruginosa* at the lowest concentration. Whereas other oils like Peppermint, clove did not show any activity even at the highest concentration [24].As essential oils can reduce biofilm formation, this data can prove a boon for treating fatal diseases where biofilm prevent disease curing [20].This study evaluates biofilm inhibitory activity of cinnamon oil along with sequel investigation on circulation pattern and antibiotic resistance profile of *P.aeruginosa.*with respect to previous study.

Different concentration 10%, 20%, 30% (v/v) of cinnamon oil was tested on strong biofilm forming MDR clinical isolates, verifying fact that increasing concentration of oil inhibit biofilm formation effectively. Ultimately hampers pathogenic strategy with enhancing drug response for antibacterial therapeutic regimen. A Brazilian study over *Cinnamomum zelyanicum* had investigated on anticandidal effects on planktonic and biofilm producing Candida species [21]. In vitro antimicrobial activity of *Cinnamon zelyanicum* (bark) against human pathogenic fungi and commensally bacteria was studied by Chaumont *et* al [31].Inhibition of biofilm was reported by Kalia *et al* using cinnamon oil in India [22]. Kim *et al*., 2015 showed cinnamon bark oil and cinnamaldehyde significantly reduced *P. aeruginosa* biofilms [23]. Many researchers have reported antibiofilm activities of cinnamon essential oil. Kerekes *et al*., 2013 used cinnamon oil against biofilms arising from mixed cultures of *E. coli* and *P. putida* and claimed to achieve complete inhibition [25].

Other plant compounds could attenuate biofilm development by inhibiting bacterial peptidoglycan synthesis [26], disrupting the permeability barrier of microbial membrane structures, causing the cell to leak out [27], modify bacterial membrane structure hydrophobicity [28],[29], or disturbing the extracellular polymeric matrix in the biofilm to release biofilm from the surface of the solid substratum [30]. Above study reveals that essential oil pose mechanism to control biofilm forming ability of *P.aeruginosa*.Further studies are obligatory to characterize the actual mode of action of anti-biofilm activity.

V. CONCLUSION

The study demonstrated cinnamon oil potential for antibacterial activity with biofilm inhibitory effect.whilst antibiotic was found to be ineffective. Present study focused on biofilms sensitivity to cinnamon oil like planktonic growth, possibly cinnamon oil possess multipotent action on controlling MDR *P.aeruginosa* along with inhibiting biofilms. Low concentration of cinnamon oil was significant, but increasing concentration to some extend is supplementary .Surveillance study is mandatory to check the circulation of Multi drug resistance *P.aeruginosa*. Use of essential oil is non-hazardous to human but highly potent against pathogens hence this property of cinnamon oil serves valuable purpose in controlling further emergence of MDR strains and its biofilm strategy. Life threatening diseases are usually associated with biofilm and drug resistance, thus by focusing root cause will accelerate new approach of remedial therapy in medical sciences.

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