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Antimicrobial Activity of *Terminalia chebula* on *Pseudomonas aeruginosa* Isolated from Nasal Secretions of Chronic Sinus Patient

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INTRODUCTION

Pseudomonas aeruginosa is a non-fermentative, gram negative bacterium that causes fatal Infections in immunocompromised patients such as victims suffering from cancer, leukamia, diabetes, burn and CF patients (Boidy et al.,1983; Vasil,1986). The viscous nasal secretions of chronic sinus patients has lead to the suspicion that *P. aeruginosa* may exist as it secretes viscous capsular polysaccharide, the alginate and also the Dnase enzyme which hydrolyses double stranded DNA, resulting in viscous nasal secretions. Therefore the objective of the study was to isolate *Pseudomonas aeruginosa* from nasal secretions of chronic sinus patients and to study various virulence contributors of this organism and to correlate the pathogenicity with the clinical symptoms. Eventually to suggest a herbal medicine for the chemotherapy of these patients, since recently the antimicrobial activity of *Rauwolfia serpentina, Allium sativum and Terminalia chebula* was assessed against several microorganisms viz, *Yersinia enterocolitica, Salmonella typhi, Salmonella typhimurium, Staphylococcus aureus,* and *Candida albicans* (Elizabeth, 2001a, b, 2002).

MATERIALS AND METHODS

Isolation of P. aeruginosa: This organism was isolated aseptically from nasal secretions of chronic sinus patient and grown over night (ON) in cetrimide broth (Hi media, Mumbai) at 37°C . Later this bacterium was confirmed by cultivating on Pseudomonas fluorescent agar (Hi Media), and on LB plates (Elizabeth,1993, 1997) which showed greenish yellow fluorescence and characteristic smell respectively. It was also confirmed by performing Gram staining, Hanging drop method, catalase, and oxidase tests (Cappuccino and Sherman,1999).

Determination of Virulence Contributors: Antibiotic Resistance: Two hundred microlitres of ON broth cultures of *P. aeruginosa* containing $1.0x10^9$ cells were spread on nutrient agar plate The anti biotic impregnated discs (Hi Media) of Ampicillin (30µg), chloramphenicol (30µg), vancomycin (30µg) and carbenicillin (100µg) were placed aseptically apart from one another on agar plate and incubated at 37°C for 18-24h. The zone of inhibition formed around the disc was measured and estimated by the formula given below. $\pi/4(D2-d2)$, where D= diameter of zone of inhibition; d= diameter of antibiotic disc.

Dnase Activity: All biochemical reactions were performed according to the method of Cappuccino et al., 1999. Briefly describing, *P. aeruginosa* was cultured on LB plate and a single colony was picked up with sterile inoculation wire loop and streaked on toluidine blue Dnase agar (Hi Media) and incubated at 37°C for 18-24 h. A white colony with pink matachromatic zone around the inoculum was scored as positive and a blue colony without any zone as negative.

Capsule Formation: It was confirmed by touching the colony with inoculation wire loop which formed a string when lifted and by capsule staining.

α-Hemolysin: A loop full of ON culture of *P. aeruginosa* was streaked on goat blood agar and incubated at 37°C for 18-24 h. Zone of inhibition along the line of inoculum with blue green colour was scored as positive for α-hemolysin production.

Phospholipase-C Activity: It was determined by line inoculation of ON cultures of this organism on egg yolk agar and incubation at 37°C for 18-24h. A clear zone of hydrolysis or inhibition surrounding the culture was scored as positive.

Proteolytic Activity: It was determined by culturing this organism by making line inoculation on milk agar plate for 18-24 h at 37°C. A line of hydrolysis in the form of clear zone around the culture was scored as positive for secretion of

proteolytic enzymes by this organism.

Catalase Activity: The ON broth cultures were tested with 3% H_2O_2 taken in a capillary tube. Extensive air bubbling in the capillary tube was scored as positive.

Oxidase Activity: The bacterium was cultured on LB plate for 18-24h. Then a colony was placed on sterile filter paper and few drops of tetra methyle para phenylene diamine dihydrochloride was added to the culture. Using sterile inoculating wire loop the cells were lysed. Appearance of purple colour was scored as positive for the presence of cytochrome oxidase activity.

Indole Test: This was done by cultivating *P. aeruginosa* in peptone water at 37°C for 24 h , later Kovac's reagent(Himedia) was added. Appearance of cherry red colour on the suface of broth cultures was scored as positive for tryptophanase activity and yellow colour as negative.

Methyle Red: Organism was cultured in MRVP broth (HIMedia) at 37°C for 24 h and few drops of methyl red was added. The appearance of red colour in the medium was scored as positive for mixed acid fermentation.

Fermentation Reactions: P. aeruginosa was cultured in peptone water containing 10% sugars such as sucrose/ mannitol / dextrose/ or lactose. A Durham tube was inserted in the tube to detect gas formation. The test tubes were incubated at 37°C for 24 h and later a few drops of red was added. Presence of yellow colour scored as positive and red colour as negative.

Antimicrobial Activity of T. chebula: T. chebula was obtained from Paderu, Visakhapatnam district, Andhra Pradesh (A.P), India. It was pulverised and fixed amount of it was mixed in particular amount of water and used as herbal stock solution by storing in refrigerator.

Disc Diffusion Method: It was done as described earlier(Elizabeth, 2001a). Two hundred microliters of ON cultures of *P.aeruginosa*, containing $1.0x10^9$ cells were spread on nutrient agar plate. Sterile paper discs of 5mm in diameter were impregnated with 2mg of *T. chebula* in 20ml volume. A positive control disc of carbenicillin (100 mg) was also included on the plate. The plates made in duplicates were incubated at 37°C for 18-24h. The zone of inhibition formed around the discs was estimated as described above.

Determination of Minimal Inhibitory Concentration (MIC): Tube dilution technique was used as previously described (Elizabeth, 2001a). Briefly describing, duplicates of serial dilutions of drug and broth were made in 1ml. Twenty microliters of inoculum containing $1.0x10^9$ cells were added into all test tubes of experimental and control (which did not receive any drug). The test tubes were incubated at 37°C for 18-24 h and the lowest concentration of the drug that inhibited the growth (showing less turbidity) was considered as minimal inhibitory concentration.

Determination of Viable Cell Counts: Two, 50ml conical flasks containing 9.9 ml LB broth were labeled as control and experimental. Each flask was inoculated with 50µl of inoculum to give $1.0x10^9$ cells per ml. The experimental flask received 500 µg / ml concentration of *T. chebula*. The flasks were incubated on orbitol shaker at 37°C. At fixed intervals the samples were taken from control and experimental flasks, serially diluted, plated on agar plates which were incubated at 37°C for 18-24h. Later the number of colony forming units (cfu) /ml were estimated.

Biochemical Alterations: The biochemical alterations induced by *T. chebula* were determined by culturing *P. aeruginosa* either in the presence of *T. chebula* at 2mg/ml concentration containing $1.0x10^9$cells/ml and/or in the absence of *T. chebula*. This herbal medicine could not induce biochemical alterations at MIC level in this organism.

RESULTS AND DISCUSSION

In the present investigation it was found that *P. aeruginosa* infects the nasal secretions of sinus patients. It was found to be gram negative, motile bacillus. It was highly resistant to the antibiotics tested such as ampicillin, vancomycin, chloramphenicol, but susceptible to carbenicillin. It produced several virulence factors (Table 1) such as Dnase, α-hemolysin, proteases, phosholipase-c, capular polysaccharide substance. It was positive to oxidase and catalase reactions. It was negative to indole, methyle red and fermentative reactions tested in the present study. The nasal secretions of chronic sinus patients are usually viscous, some times with blood streaks. This may be due to the viscous polysaccharide capsular material and Dnase secreted by *P. aeruginosa* which hydrolyses the DNA of cells eventually resulting in killing of host cells and high viscosity. α-

hemolysin is a cytotoxin, another virulence factor secreted by this organism which partially destroys RBC of the host, resulting in the presence of blood in nasal secretions. The proteases and lipases produced by this organism may help this organism to invade the host tissue and evade the host immune responses. The disc diffusion method showed that *T. chebula* was effective against this organism as it formed a zone diameter of 50mm at 2mg concentration and carbenicillin (100µg) showed 32mm suggesting that *P.aeruginosa* was sensitive to *T. chebula*. The MIC of this herbal medicine was 500µg towards $1.0x10^9$ cells of *P. aeruginosa*. The drug treated organisms were sluggish when compared to the untreated controls. The viable cell counts (Fig. 1) of *P. aeruginosa* in the presence of this herbal medicine

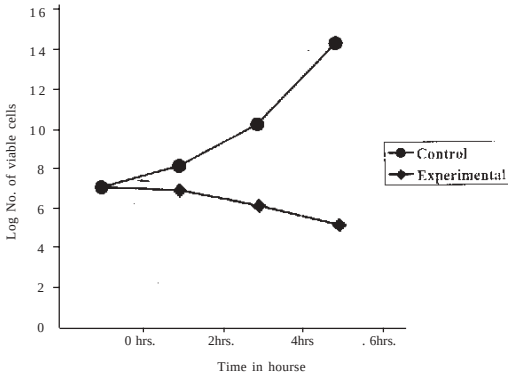

Fig. 1. Effect of *T. chebula* on *P. aeruginosa*

Table 1: The antimicrobial activity of *T. chebula* and biochemical alterations in *P. aeruginosa*

S.No.	*Test*	*Control*	*Experimenta*
1.	Gram staining	gram negative	gram negative
2.	motility	less motile	actively motile
3.	Dnase	+	-
4.	hemolysin	+	-
5.	phospholipase-c	+	-
6.	proteases	+	-
7.	catalase	+	+
8.	oxidase	+	-
9.	indole	-	-
10.	methyle red	-	-
11.	Sucrose fermentation	A-/G-	A-/G-
12.	dextrose	,,	,,
13.	lactose	,,	,,
14.	mannitol	,,	,,

at MIC showed that there was 2 log cycle reduction by 6th hour suggesting that *T.chebula* has bacteriostatic effect on *P. aeruginosa*. *T. chebula* could not produce any biochemical alterations at MIC. But when tried at higher concentration of 2mg/ml against $1.0x10^9$cells , there were several alterations suggesting that *T. chebula* has strong antimicrobial activity against *P. aeruginosa* (Table 1).

CONCLUSION

These results clearly indicate that *P. aeruginosa* infects the synovial fluid of chronic sinus patients. It secreted several virulence factors such as β-lactamase, viscous capsular polysaccharide material, Dnase, α- hemolysin, proteases and phospholipase-c. These factors may be responsible for the symptoms exhibited by these patients. *P.aeruginosa* was sensitive to *T. chebula* which has a MIC of 500µg, a bacteriostatic agent and induced biochemical alterations in this organism.

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KEY WORDS *P. aeruginosa*. Chronic Sinus Patient. Isolation. Antimicrobial Activity. *T. Chebula,*

ABSTRACT *Pseudomonas aeruginosa* was isolated from the nasal secretions of chronic sinus patient, using a selective medium the cetrimide broth, and confirmed by culturing on Pseudomonas fluorescent medium. This organism was highly resistant to antibiotics such as ampicillin, chloramphenicol, vancomycin 30mg each but sensitive to carbenicillin(100mg) which showed a zone diameter of 32 mm. This organism has been biochemically characterized as α-hemolytic, Dnase positive, proteolytic, phospholipase-c positive, catalase, oxidase, nitrate reductase positive, but negative for indole and fermentative reactions of sucrose, mannitol, dextrose and lactose. *Terminalia chebula*, commonly known as myrobalam was found to be effective against this microorganism, as it showed a zone diameter of 50 mm with 2 mg concentration as determined by disc diffusion method. The minimal inhibitory concentration (MIC) of *T. chebula* against $1.0x10^9$ cells of *P. aeruginosa* was 500 µg as determined by the tube dilution technique. The viable cell counts of *P. aeruginosa* in the presence of *T. chebula* at MIC showed reduction of 2 log cycles by 6 h of incubation suggesting that this herbal medicine is bacteriostatic in nature. The various virulence contributors of *P. aeruginosa*, such as high antibiotic resistance, hemolysin, phospholipase-c, Dnase and

proteases were inhibited by *T. chebula* at a concentration of 2 mg/ml. These results clearly show that *P. aeruginosa* infects the nasal secretions of sinus patients and is one of the responsible organisms of the pathogenic traits exhibited by these victims. This infection can be controlled by *T. chebula*.

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