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Isolation and Evaluation *of In Vitro* Antibiofim Activity of *Vitex negundo* L. Essential Oil

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ABSTRACT

In vitro antibiofilm activity of essential isolated from *Vitex negundo* L. leaves was studied using four different human pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Shigella dysenteriae*. Powder of dried leaves of *V. negundo* were taken for isolation of essential oil by hydro distillation method using Clevenger apparatus. Initially, the minimum inhibitory concentration (MIC) of oil was determined against bio films produced by four different human pathogens. Then taking the oil with MIC, percentage of bio film destruction and viable cells were determined using specific bio film formation (SBF) assay. For 100 g dried leaves, 1.0 % v/w essential oil was obtained. The minimum inhibitory concentration for bio film destruction was found to be 0.06 ml essential oil. During SBF assay, among the four species, *P. aeruginosa* showed maximum biofilm destruction (86%) followed by *S. dysenteriae* which showed 81% bio film destruction. The remaining two strains i,e *S. aureus* and *E. coli* showed 77% and 75% destruction, respectively. Maximum percentage of viable cells observed for *E.coli* (25%) followed by *S. aureus* (23%). The remaining two strains *S. dysenteriae* and *P.aeruginosa* showed 19% and 14%, respectively. Five parameters measuring biomass (Maximum thickness [µm], substratum coverage [%], roughness coefficient, average diffusion distance and surface to bio-volume ratio [$\mu m^2/\mu m^3$]), analyzed by COMSTAT software and found that the resulting values were significantly lesser for bio films exposed essential oil than control. The results obtained in this study proves that the potent efficacy of *V. negundo* essential oil for the control of bio film formed by the studied organisms.

KEYWORDS: *Vitex negundo*, Essential oil, Antibiofilm activity, Minimum Inhibitory Concentration, Specific Biofilm Formation assay.

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INTRODUCTION

Bacteria form complex multicellular structures called biofilms[1]. Bio film formation is commonly considered to happen in four main stages: (1) bacterial attachment to a surface, (2) micro colony formation, (3) bio film maturation and (4) detachment of bacteria which may then colonize new areas[2]. Bacteria within the bio film, termed sessile bacteria, exist in a stationary or dormant growth phase[3] and exhibit phenotypes that are distinct from plank tonic bacteria[4]. Bio film allied cells have the ability to adhere irreversibly on a wide variety of surfaces, including living tissues and indwelling medical devices as catheters, valves, prosthesis and so forth[5]. They cause persistent chronic and recurrent infections and are highly resistant to antibiotics and host immune defences[6]. Bio film resistance is due to numerous reasons, like restricted diffusion of antibiotics into bio film matrix, expression of multidrug efflux pumps, type IV secretion systems, decreased permeability and the action of antibiotic-modifying enzymes[7]. The increased bio film resistance to conventional treatments enhances the need to develop new control strategies[8]. In recent years, search has been started towards naturally occurring compounds of plant origin capable of blocking bio film formation[9].

Extracts and essential oils from a wide range of medicinal plants have been evaluated for antibiofilm activity. For example, bio film inhibitory nature of plant extracts (solvent extracts and fractions) has been reported against *E. coli* [10,11], *Listeria monocytogenes*[12], *S. aureus*[10,13] and *Candida albicans*[14]. Several studies have reported the antimicrobial properties of essential oils[15]. They comprise complex and heterogenous mixtures of substances comprising several classes with diverse biosynthetic origin. The main group includes terpenes (monoterpenes, sesquiterpenes) and terpenoids together with aromatic (phenylpropanoids) and/or aliphatic compounds[16-18]. Essential oils exert low toxicity in mammalians and degrade quickly[19]. In recent years, studies on the antibiofilm activity of essential oils have been increased. Anti-bio film activity of essential oils has been reported against *S. aureus* using *Nigella sativa* seed oil[20], lemongrass oil[21], carvacrol and thymol[22,23], oregano oil[24], cassia, Peru balsam and red thyme essential oils[19], tea tree oil[25] and lavender and Melissa oil[26].

Vitex negundo L. is a woody, aromatic shrub belonging to Verbenaceae which bears tri or penta foliate leaves on quadrangular branches with bluish-purple coloured flowers in branched tomentose cymes. It is found in humid places or along water courses in wastelands and mixed open forests and has been reported to exist in Afghanistan, India, Pakistan, Sri Lanka, Thailand, Malaysia, eastern Africa and Madagascar[27]. The plant has anti-inflammatory, antifungal, antibacterial and analgesic properties. It is used in the treatment of superficial bruises, injuries, sores and skin infections as traditional medicines. Leaf oil increases hair growth and brain function. Roots play essential role in rheumatism, dyspepsia, piles etc. [28,29]. Although all plant parts are used, but the

leaves and root extract represent more significant medicinal activity[30]. Recently, the composition of the essential oil of *V. negundo* was determined by GC-MS analysis[31]. Out of six compounds, caryophyllene (21.58 %) and epiglobulol (47.13 %) are found to be the major compounds. The molecular docking studies was also carried out using these constituents against some bacterial proteins and showed caryophyllene and epiglobulol have very good docking scores which in turn supporting the antibacterial potential of this oil. However, there is a lack of information in the literature regarding its *in vitro* antibiofilm activity. Therefore, this study was designed to evaluate the oil isolated from *V.negundo* using four bio film forming bacteria such as *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Shigella dysenteriae*.

EXPERIMENTAL

Fresh leaves collected from *Vitex negundo* L. trees which are grown in Bharathiar University Campus, Coimbatore, Tamil Nadu, India. The plant was authenticated by Botanical Survey of India, Southern Regional Centre, TNAU, Coimbatore. Physiologically mature and disease free leaves were thoroughly washed with running tap water and kept in the oven for two days at 50^{o}C. The dried leaves were powdered using Wiley Mill Grinder. The 100 g leaf powder was placed in a 1 liter round bottomed flask containing 300 ml distilled water and oil was extracted using a Clevenger apparatus by hydro-distillation method. In this study, anti-biofilm activity of the extracted oil of *V.negundo* was assed against one gram positive (*Staphylococcus aureus*) and three gram negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella dysenteriae*) human bacterial pathogens.

DETERMINATION OF MIC

To determine MIC of oil, 5 ml screw capped tubes containing 3 ml of LB medium and plant extracted oil at various concentrations (0.02, 0.04, 0.06, 0.08 and 0.1 ml) were inoculated with 30 µl of each microbial cells in the log phase of growth. For each concentration tested, tubes were prepared in triplicate along with control and incubated in an orbital shaker (200 rpm). Growth was determined turbidometrically (600 nm) at the initiation of the experiment and after 1.5 h (*E.coli* and *S.aureus*), 2.5 h (*P.aeruginosa*) and 3.5 to 4 h (*S. dysenteriae*). The extracted oil was diluted in methanol (20% v/w) prior to use.

SPECIFIC BIOFILM FORMATION ASSAY

For specific biofilm formation assay, each bacterial culture was grown in 5 ml culture tubes containing 2 ml of LB medium with 0.06 ml of essential oil. Three identically prepared tubes were used for each bacterial culture. Three of the nine tubes were used to measure growth in suspended culture (G Tubes -20 µl bacterial culture + 2 ml LB; G tubes denote the optical density of the

bacterial cells), three tubes were used to measure bio film growth (B tubes - 20µl bacterial culture+125µl crystal violet (0.3%) + 2 ml LB; B tubes denote the amount of bio film formed) and three tubes served as controls for abiotic factors (NC tubes - 125µl crystal violet (0.3%) +2 ml LB; NC tubes are the amount of crystal violet that adhered to test tubes due to abiotic factors). Each bacterial culture was grown to the late log phase and all tubes were incubated in an orbital shaker for 17±1h. Following incubation, cells in the G tubes were mixed well and OD was taken at 600 nm. Then the suspended culture was poured out and the tubes were rinsed well 4 or 6 times with distilled water. Any remaining crystal violet was dissolved in 2 ml of an ethanol-acetone (80:20) solution and the absorbance at 570 nm of each resultant solution was measured spectrophotometrically. Specific bio film forming ability of each microorganism was calculated using the following formula:

SBF = B – NC/G

STATISTICAL ANALYSIS

COMSTAT is a computer program that analyzes the images of bio films taken by microscopes by applying variables such as total biomass, maximum thickness, substrate coverage, roughness co- efficient, average diffusion distance and surface to bio-volume ratio[32].

RESULTS AND DISCUSSION

In the present investigation, an attempt was taken to extract essential oil from dried leaves of *V.negundo*. When dried 100g leaf samples were subjected to hydro-distillation for 4 hours using Clevenger apparatus, 1ml (1.0 % v/w) pale yellow coloured oil was obtained. This is contrary to the report of [33], who extracted only 0.1% (v/w) from this leaves. The influence of location on various agronomic characteristics of oil bearing plants were also reported for many plants[34-38].

Different concentrations of oil were screened to determine its ability to inhibit the growth of each microorganism. Out of six concentrations, 0.06 ml was required to obtain 50% of growth of all the tested bacterial strains (fig.1). This result is in agreement with the observations made by others[39-41].

Table 1. Percentage of bio film destruction and viable cells

S.No	Name of the organism	Difference in OD (SBF=B-NC/G)	Percentage of bio film destruction	Percentage of viability
1	*E. coli*	75%	0.75	25%
2	*P. aeruginosa*	86%	0.86	14%
3	*S. aureus*	77%	0.77	23%
4	*S. dysenteriae*	81%	0.81	19%

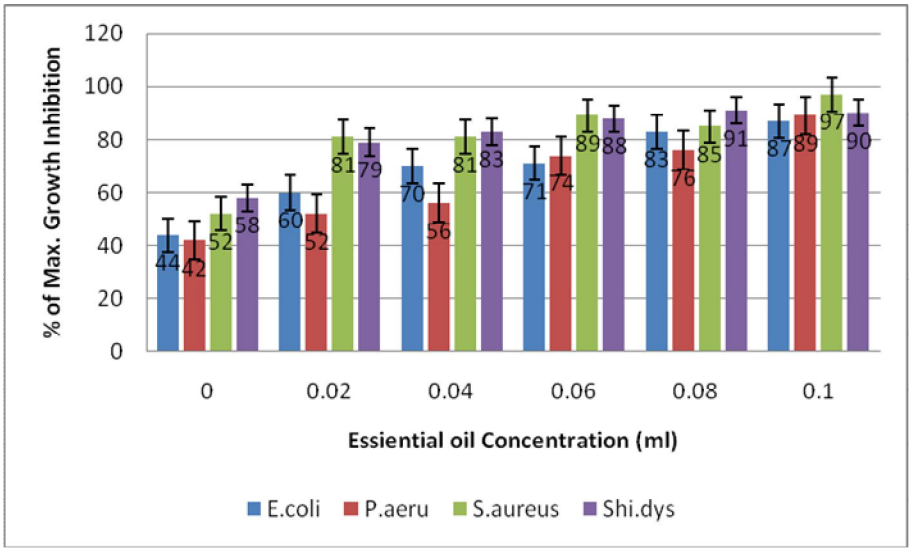

Fig.1: Minimum inhibitory concentration of essential oil

The SBF assay was conceived as a simple crystal violet based approach to find out anti-biofilm property of isolated oil. During this assay, biofilm formation capacity of four species was observed and calculated. Among them, *P. aeruginosa* showed maximum biofilm destruction (86%) followed by *S. dysenteriae* which showed 81% biofilm destruction. The remaining two strains i.e. *S. aureus* and *E. coli* showed 77% and 75% destruction, respectively. After destruction, the percentage of viable cells has also been calculated. Maximum percentage of viable cells observed for *E.coli* (25%) followed by *S.aureus* (23%). The remaining two strains *S.dysenteriae* and *P.aeruginosa* showed 19% and 14% respectively (Table 1). These results confirm previously published reports[42, 39].

COMSTAT is a computer software which is used to quantify biofilm structure by applying different variables such as maximum thickness (µm), substratum coverage (%), roughness coefficient, average diffusion distance and surface to bio-volume ratio ($\mu m^2/\mu m^3$). These variables were selected because they are quite easy to interpret in biological and physical terms. Mean thickness suggests the spatial dimensions of the bio film, roughness represents a measure of bio film heterogeneity, substratum coverage reflects how efficiently the strain colonizes the substratum and surface to volume ratio tells how large a portion of the bio film is exposed to the nutrient flow. In the present investigation, these parameters were found significantly lesser for bio films exposed to essential oil than control. And also results obtained were strongly agreed with the trend showed by the SBF assay (Table 2). The changes in the all six parameters of treated biofilms and untreated (control) are compared for four bacterial strains. It was found that *P.aeruginosa* showed less biomass (1.60±0.4) followed by *S. aureus* (1.92±0.06). Less thickness of bio film was shown by *S. dysentriae* and less substratum coverage shown by *P.aeruginosa*. Roughness coefficient and average diffusion distance

were found to be low for *E. coli* compared to other strains. Among four strains, *S. aureus* only showed low surface to bio volume ratio (2.0±0.02). Attempts were made to describe biofilm structures quantitatively include all these five parameters using four bacterial monospecies such as *Pseudomonas putida, P. aureofaciens, P. fluorescens* and *P. aeruginosa*[33].

Table 2. Comstat analysis of various bio film components formed by microorganisms

Name of the bacteria		Number of the sample	Total biomass (μm³/μm²)	Maximum thickness (μm)	Substratum coverage (%)	Roughness coefficient	Average diffusion distance (μm)	Surface to bio volume ratio (μm²/μm³)
Escherichia coli	Control (LB+ *E.coli*)	1	4.5±0.8[a]	42.2±2.1[a]	40±15[a]	3.2±0.8[a]	0.04±0.02[b]	4.56±0.5[a]
	E.Coli + Plant essential oil+ LB	9	**2.01±0.02**[b]	**28.5±2.0**[b]	**30±2**[b]	**0.9±0.3**[b]	**0.09±0.02**[a]	**2.8±1.0**[b]
Pseudomonas aeruginosa	Control (LB+ *P. aeruginosa*)	1	3.98±0.2[a]	40.2±2.0[a]	29.5±8[a]	4.76±0.7[a]	0.87±0.2[a]	4.95±0.4[a]
	P. aeruginosa + Plant essential oil + LB	9	**1.60±0.4**[b]	**20.02±1.0**[b]	**19±5**[b]	**2.1±0.5**[b]	**0.18±0.18**[b]	**2.5±0.5**[b]
Staphylococcus aureus	Control (LB+ *S. aureus*)	1	2.65±0.01[a]	46.05±2.8[a]	47±8[a]	4.8±0.2[a]	0.8±0.1[a]	4.7±0.02[a]
	S. aureus + Plant essential oil+LB	9	**1.92±0.06**[b]	**21.06±1.07**[b]	**21±2**[b]	**1.4±0.6**[b]	**0.19±0.09**[b]	**2.0±0.02**[b]
Shigella dysenteriae	Control (LB+ *S. dysenteriae*)	1	3.9±0.04[a]	39.02±2[a]	46±0.5[a]	4.0±0.6[a]	2±0.4[a]	6±0.2[a]
	S. dysenteriae + Plant essential oil+ LB	9	**2.02±0.06**[b]	**19.06±4.02**[b]	**23±1.5**[b]	**1.5±0.2**[b]	**0.5±0.07**[b]	**3.02±0.5**[b]

Biofilms were grown on microtitre plates. Essential oil was added and resulted in inhibition of biofilm formation. All values are means ± standard deviations.

a. Biofilm formation was significantly greater ($p<0.05$) for the LB medium control
b. Biofilm formation was significantly lesser ($p<0.05$) for essential oil

CONCLUSION

In the present investigation, anti-biofilm activity of *V.negundo* essential oil was determined using four bacterial species. The results obtained in this study suggest the potent efficacy of *V.negundo* essential oil in prevention and control of biofilm associated microbial infections and diseases. And also the present investigation appears to be the first report of the *in vitro* antibiofilm activity of essential oil of this species.

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