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Research Article

ANTI BIOFILM EFFECT OF MEDICINAL PLANT EXTRACTS AGAINST CLINICAL ISOLATE OF BIOFILM OF *ESCHERICHIA COLI*

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ABSTRACT

Objective: The objective of the present study is to evaluate anti biofilm effect of both the aqueous and chitosan coated extracts of *Azadirachta indica, Vitex negundu ,Tridax procumbens* and *Ocimum tenuiflorum*i was evaluated against biofilm development of clinical isolate of *E.coli*

Methods: Crude aqueous and chitosan extracts of respective plants with different concentration was evaluated against biofilm adopting microtitre plate crystal violet assay. Biofilm matrix was extracted from the respective treatment and the biochemical composition mainly total carbohydrates and total protein was studied.

Results: Biofilm inhibition study revealed both free and chitosan coated plant extracts inhibited biofilm formation, enhanced effect on biofilm inhibition was recorded in polymer coated extracts of the all the tested plants. Biochemical composition of biofilm matrix mainly total carbohydrates and total protein was highly reduced in all the tested concentration of polymer coated plant extracts.

Conclusion: Anti biofilm effect of plant extracts coated with biocompatible polymer chitosan would suggests the possible utilization of the extracts as the effective anti bacterial agents against pathogenic bacteria.

Keywords: Biofilm, *E.coli*, Plant extracts, Chitosan, Matrix, Inhibition

INTRODUCTION

Biofilms - adherent communities of bacteria surrounded by a matrix of extracellular polymeric substance (EPS) - are the prevailing microbial lifestyle in the environment [1]. The role of the biofilm is to attach to abiotic surfaces, the epithelia of multicellular organisms, and interfaces such as that between air and water [2]. Surface adhesion of bacteria is an essential step and is required for the bacteria to arrange themselves favourably in their environment [3].Some bacterial biofilms have been reported to have useful effects on food chains, sewage treatment plants, to eliminate petroleum oil/hydrocarbon spillage from the oceans [4]. Now the biofilm is considered as major target for the pharmacological development of drugs. A biofilm serves to promote bacteria persistence by resisting antibiotic treatment and host immune responses [5]. Antibiotics are rendered ineffective when biofilms form due to their relative impermeability, the variable physiological status of microorganisms, subpopulations of persistent strains, and variations of phenotypes present [6]. Biofilms have been reported to show increased resistance to antimicrobial agents including antibiotics compared to free-floating cells [7]. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illnesses since ancient times [8]. Modern science and technological advances are accelerating the discovery and development of innovative pharmaceuticals with improved therapeutic activity and reduced side-effects from plants. Plant compounds are widely accepted due to the perception that they are safe and they have a long history of use in folk medicine as immune boosters and for the prevention and treatment of several diseases [9]. Over the years, the use of medicinal plants, which forms the backbone of traditional medicine, has grown with an estimated 80% of the populations, mostly in developing countries, relying on traditional medicines for their primary health care [10]. Plant-derived substances under intensive research for possible applications in the pharmaceutical industry include crude extracts of leaves, roots, stems and individual compounds isolated from these, essential oils and essential oil components [11].Although a lot of research on plants and the active constituents is currently underway, the focus is mainly on the antimicrobial properties against planktonicor biofilm forming bacteria [12]. .In the present study, anti biofilm effect of biocompatible polymer coated aerial parts extracts of *Azadirachta indica, Vitex negundu ,Tridax procumbens* and *Ocimum tenuiflorum*i against clinical isolate of *E.coli* biofilm was discussed.

MATERIALS AND METHODS

Plant materials

Healthy and fresh leaves of *Azadirachta indica, Vitex negundu ,Tridax procumbens* and *Ocimum tenuiflorum*i were collected from the respective plants grown in home garden.Collected materials were washed in tap water followed by successive washing in distilled water. Washed materials were shade dried. Dried material was homogenized in domestic mixture into fine powder, stored in plastic container at room temperature used for further studies.

Crude extraction

Crude extraction of the respective plant material was carried out by extraction of the dried materials [13] .The dried powder of respective plant material (50 g) was soaked separately with 250 mL of methanol in a 500 mL conical flask for 48 hours at room temperature, without shaking. The solvent was filtered through Whatman filter paper No. 1 and concentrated on a rotary vacuum evaporator. Concentrated crude extract was reconstituted in dimethyl sulfoxide (DMSO) to make a stock solution of 10,25,50,75 and 100 g/mL and stored at−20 °C until use.

Preparation of biocompatible polymer chitosan coated extracts

Chitosan was obtained from Rolex chemical industries, Mumbai and refined twice by dissolving it in dilute HOAc solution. The solution was filtered, the chitosan was precipitated with aqueous sodium hydroxide, and the precipitate was dried in vacuum at room temperature [14].The degree of deacetylation was about 85% as determined by elemental analysis, and the average molecular weight of the chitosan was 220kDa as determined by viscometric methods [15]. Respective plant extracts with respective concentration was mixed with 1% pre treated chitosan solution under magnetic stirrer for 3 hours at 35ºC. The slurry thus obtained was dried at 40ºc in a hot air oven for 12 hours, dried material was reconstituted to make a stock solution of 10,25,50,75 and 100 g/mL and used for further study.

Biofilm inhibition study

Bacterial strain and growth condition

Clinical isolate of *E.coli* obtained from Madurai Medical college hospital, Madurai, Tamil Nadu, India. The strain was isolated from patient with severe urinary tract infection and maintained on slope

of Tryptic soy agar slant.Tryptic soy broth (Hi media) was used for inocula preparation of the bacterial strain. Cultures was inoculated from fresh slopes and incubated with shaking at 37° C for 24 hours. Cells were collected by centrifugation and the collected cell debris washed twice in PBS and suspended to OD_{520} prior to use in biofilm experiments.

Biofilm inhibition assay

Biofilm inhibition carried out in 96 wall plates adopting modified method of biofilm inhibition spectrophotometric assay [16] .100µl of cell suspension of *E.coli* thus prepared was added into 96 well titre plate and different concentration of free and polymer coated plant extracts as 25, 50, 75 and 100 µg /ml was added and incubated at 37º C for 3 days. After the incubation, the liquid suspension was removed and 100 µl of 1% w/v aqueous solution of crystal violet was added. Following staining at room temperature for 30 minutes the dye was removed and the wells were washed thoroughly, 95% ethanol was added and incubated for 15 minutes. The reaction mixture was read spectrophotometrically at 570nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula

$$\% \text{ of inhibition} = \frac{\text{OD in control} - \text{OD in treatment} \times 100}{\text{OD in control}}$$

Scanning electron microscopy (SEM)

Biofilms were examined by SEM after processing of samples by a freeze-drying technique [17]. .Biofilms were fixed with glutaraldehyde (2.5%, v/v, in 0?1 M cacodylate buffer, pH 7?0), washed gently three times in distilled water, and then plunged into a liquid propane/isopentane mixture (2 : 1, v/v) at 2196 µC before freeze-drying under vacuum (1026 torr, 1.361024 Pa). Samples were finally coated with gold and palladium and viewed under a Carl zeiss subra (Germany)scanning electron microscope.

Effect of free and polymers coated plant extracts on the biochemical composition of biofilm matrix

Isolation of biofilm matrix

Biofilm matrix material was isolated to study the biochemical composition by the standard method .Adherent biofilms were transferred to screw cap bottles containing 10 ml distilled water. The bottles were sonicated for 5 min in an ultrasonic water bath and vortexed vigorously for 1 min to disrupt the biofilms. Cell suspensions were then pooled and centrifuged. The collected supernatant used as source for studying biochemical composition mainly protein by Lowry *et al* and total carbohydrate by Dubois *et al* [18]

RESULT

Biofilm inhibition studies carried out using plant extracts and polymer coated extracts at all the tested concentration have successfully inhibited biofilm formation of *E.coli* All the plant extracts tested inhibited biofilm as dose dependent manner .But significant effect was recorded in polymer coated extracts ($p>0.05$). The results in Table 1 clearly indicate the enhanced antibiofilm effect of free and chitosan coated plant extracts in microtitre plate assay. Free extracts of *A.indica* with 10,25,50 and 75 µg/ml recorded 22.0,34.5,47.5 and 59.0% of biofilm inhibition.

Table 1: Shows

S. No.	Tested Plants	Biofilm inhibition (%)				
		10	25	50	75	100
1.	*Azadirachta indica*					
	FPE	22.0	34.5	47.5	59.0	65.0
	CPE	60.0	71.2	82.3	91.0	100.0
2	*Vitex negundu*					
	FPE	12.0	32.0	43.0	51.0	65.0
	CPE	59.0	65.0	79.0	87.0	100.0
3	*Tridax procumbens*					
	FPE	12.0	21.0	30.0	37.6	41.9
	CPE	27.0	33.2	41.2	51.0	60.0
4	*Ocimum tenuiflorum*i					
	FPE	10.0	16.2	23.4	29.0	33.0
	CPE	21.3	30.1	40.3	50.3	60.1

FPE-free plant extract; CPE-chitosan coated plant extract

Table 2:

S. No.	Tested Plants	Concentration				
		100	75	50	25	10
1.	*Azadirachta indica*					
	CPE(TP)	11.1	17.2	31.4	42.0	51.2
	FPE(TP)	21.2	30.1	42.0	63.2	71.2
	CPE(TC)	21.0	25.0	39.6	41.2	65.0
	FPE(TC)	30.4	40.2	48.1	53.2	60.1
2	*Vitex negundu*					
	CPE(TP)	19.8	22.8	35.7	55.6	65.4
	FPE(TP)	34.2	50.1	61.0	72.0	80.5
	CPE(TC)	29.0	31.2	43.1	52.0	60.1
	FPE(TC)	37.4	41.2	56.0	65.2	70.1
3	*Tridax procumbens*					
	CPE(TP)	34.5	45.4	51.2	70.2	80.4
	FPE(TP)	45.0	53.2	60.2	80.1	90.2
	CPE (TC)	46.0	52.0	60.1	68.2	71.0
	FPE (TC)	56.0	65.4	74.2	82.0	91.0
4	*Ocimum tenuiflorum*i					
	CPE(TP)	42.0	50.0	60.2	80.2	91.2
	FPE(TP)	36.0	50.0	70.1	90.4	123.1
	CPE(TC)	52.1	63.0	70.2	89.2	91.0
	FPE(TC)	72.0	80.0	89.3	92.3	99.0

FPE-free plant extract; CPE chitosan coated plant extract; TP- (Total protein in mg); TC (Total carbohydrate in mg)

While 100 µg/ml of free *A.indica* resulted in 65 % inhibition, the same concentration of chitosan coated extracts (CPE) brought about complete inhibition of the biofilm(100%).Similar improved anti biofilm effect was recorded in remaining concentration. Followed by *A.indica*, *V.negundo* free extract recorded maximum biofilm inhibition of 65 and 51% at 100 and 75 µg/ml. (43,32 and 12% of biofilm inhibition was recorded at 50,25 and 10 µg concentration). But the chitosan coated extract revealed 90,87,79,65 and 59% of inhibition. Free *Tridax procumbens* and *Ocimum tenuiflorum*i showed least biofilm inhibition which revealed 12.0,21.0,30.1,37.6,41.9% and 10.0,16.2,23.4,29.0,33.0% of biofilm inhibition at the respective concentration..However, increased biofilm inhibition was brought about by chitosan coated extracts. 27.0,33.2,41.2,51.0,60.0 % and 21.3,30.1,40.3,50.3,60.1% was recorded in chitosan coated *Tridax procumbens* and *Ocimum tenuiflorum*i. Scanning electron microscopy was carried out with the treatment which recorded high anti biofilm effect. Scanning electron microscopy of the biofilm derived from chitosan coated *A.indica* extract treatment reveals degeneration of biofilm with weakened cell masses (Figour 1a) while the control exposed compact tightly packed cell aggregates (Fig. 1b). Biochemical composition of the biofilm matrix was highly reduced in the both free and polymer coated extracs. Table 2 shows the gradual reduction of total carbohydrate and protein with the increasing concentration of free and chitosan coated plant extracts. Though, all the tested free and chitosan coated plant extracts reduced total carbohydrate and total protein, maximum reduction of carbohydrate and protein was recorded in all the tested concentration of chitosan coated extracts of *A.indica* followed by *V.negundo* (Table 2).Maximum reduction of total carbohydrate and protein was observed in chitosan coated extracts with all the tested concentration. Gradual reduction of total protein and total carbohydrate was recorded in increasing concentration of free and chitosan coated plant extracts .

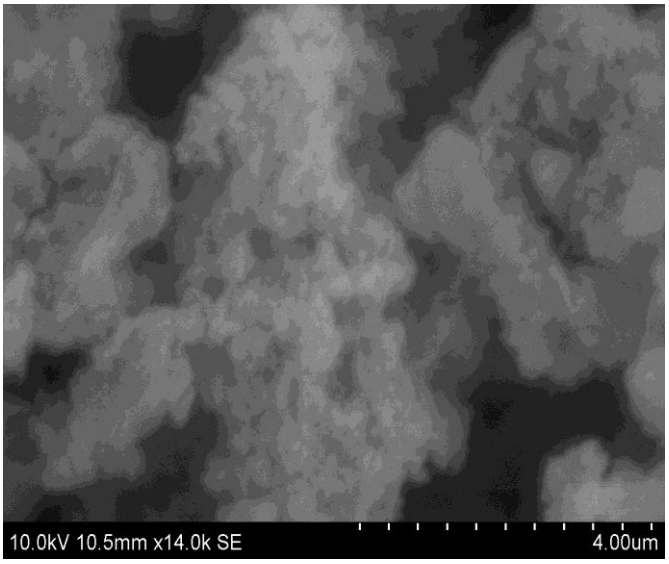

Fig. 1a: Scanning electron microscopic image of biofilm of *E.coli*

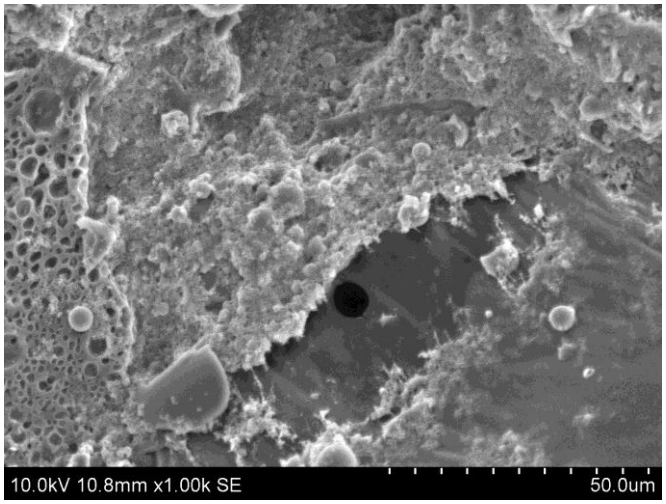

Fig. 1b: Scanning electron microscopic image of biofilm of *E.coli* treated with chitosan coated extract of *A.indica*

DISCUSSION

Due to the increase in complexity of most microbial infections and the resistance to conventional therapy, researchers have been prompted to identify alternatives for the treatment of infections. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illness since ancient times [19]. In the present study, anti biofilm effect of plant extracts against *Staphylococcus aureus* has been studied adopting biofilm inhibition spectrophotometric assay. All the plant extracts tested inhibited biofilm as dose dependent manner. Anti biofilm effect of various plant extracts against biofilm of human pathogenic bacteria has been reported by workers [20,21,22].

However, anti biofilm effect of plant extracts coated with natural biocompatible polymers has not been studied. Chitosan a natural polymer has been reported as a polymer-based protective agent to

stabilize the various active compounds including nanoparticles [23]. Because of the biocompatibility, biodegradability, nontoxicity and adsorption properties of chitosan, it was used as a stabilizing agent to prepare Ag, Au and Pt nanoparticles. These chitosan-protected bioactive compounds can be easily integrated into systems relevant for pharmaceutical, biomedical, and biosensor applications. Therefore, it has attracted considerable interest due to its medicinal properties, such as antifungal, antibacterial, antiprotozoan, anticancer, antiplaque, antitartar, hemostatic, wound healing and potentiates anti-inflammatory response, immunopotentiation, antihypertensive, serum cholesterol lowering,immune enhancer, increases salivary secretion (anti-xerostomial) and helps in the formation of bone substitute materials [24]..Hence an attempt has been made to study the anti biofilm effect of chitosan coated plant extracts against biofilm of *Staphylococcus aureus*. Chitosan coated extracts with all the tested concentration revealed enhanced anti biofilm activity. The increased antibiofilm effect of chitosan coated plant extracts may be due to the inhibition of exopolysaccharide synthesis limiting the formation of biofilm [25] or due to diffusion of CS-plant extracts through the channels present in the biofilms followed by the sustained release of phytochemicals in the respective plant extracts which may then impart antimicrobial function.

Biochemical composition of the biofilm matrix has been highly reduced. The matrix is one of the most distinctive features of a microbial biofilm. It forms a three dimensional, gel- like, highly hydrated and locally charged environment in which the microorganisms are largely immobilized. Matrix-enclosed micro colonies, sometimes described as stacks or towers, are separated by water channels which provide a mechanism for nutrient circulation within the biofilm. The composition of the matrix varies according to the nature of the organism and reduction in the biochemical composition of the biofilm matrix leads to weakening of the biofilm thus facilitating the entry of the drugs [3]. Purification and identification of the phytochemicals responsible for the anti biofilm effect and the coating of the bioactive compound with chitosan will lead to development of effective anti microbials against harmful pathogenic microorganism

CONCLUSION

Particular attention is oriented nowadays towards the need for antimicrobial textiles and polymers that are able to reduce or eliminate infections completely; especially those caused by antibiotic-resistant bacterial strains by forming some specific virulent factors.Biofilm is the one of the major virulent factor of most of the pathogenic microorganism Therefore, the developments of effective and safe medicine particularly plant extracts with antimicrobial properties have recently received growing interest from both academic and industrial sectors. The present study demonstrated the effective biofilm inhibition of *E.coli* by plant extracts coated with biopolymer.Further study will helpful to understand molecular mechanism of anti biofilm effect of chitosan coated extracts.

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