ANTIBIOFILM FORMING ACTIVITY OF NATURALLY OCCURRING COMPOUND

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ABSTRACT

Introduction: It has been observed that people in general do not pay much attention to maintain proper oral hygiene as a result organisms which are a part of normal flora of oral cavity form oral biofilm over the teeth. When patient is catheterised mostly the catheter remain there for a long period especially in older immobilised very sick patients and over these catheter due to fecal contamination ecoli biofilm formed. Biofilm forming property confers virulence characteristics thus making such bacteria highly resistant to commonly used antibiotics. We analysed the antibiofilm activity of natural occurring substances like dandasa (cassia fistula) and green tea (Camellia sinensis).

Material and Methods: Forty streptococcus mutans and 45 ecoli samples were taken from oral biofilm and contaminated urinary catheter respectively and characterised using conventional biochemical, cultural and molecular methods. The biofilm forming activity of these isolates using 96 well plates were checked. Then the dandasa and green tea with those biofilm formers were mixed and observed the antibiofilm forming activity.

Results: Both dandasa and green tea in a concentration of 6.2 mg/ml and 12.5 mg/ml respectively show good antibiofilm forming activity against streptococcus mutan and in a concentration of 12.5 mg/ml and 3.1 mg/ml respectively against E. coli. Combinations of dandasa with green tea were found to be not more effective in inhibiting biofilm formation suggesting in different activity with anti-adhesive index of 1.5 and 0.75 against Streptococcus mutans, and ecoli respectively.

Conclusion: Streptococcus mutans in oral biofilm and ecoli in urinary catheter biofilm exhibited biofilm formation which is the cause for antibiotic resistance and providing shelter to other organism. Green tea and dandasa provide a good antibiofilm activity individually.

INTRODUCTION

Biofilms

Biofilms are irreversibly attached to a substratum and to each other.[1] Biofilms are microbial communities that exist on a biotic and sometimes biotic surfaces[2] and can be difficult to control since they can form where cleaning is not performed properly. Recent research indicates that biofilms can exist as a mass of microcolonies in a single layer or with vertical and horizontal channels allowing liquid flow and dispersion of nutrients and waste components.[3] These biofilms could serve as a source of product contamination[4] and may be reservoir for pathogenic or spoilage bacteria.[5] Oral biofilm (Dental plaque) is a soft deposit that accumulates on the teeth. In addition to the bacterial cell, plaques contain a small number of epithelial cell, leukocytes and macrophages.

Streptococcus mutans

Streptococcus mutans is Gram – positive, found in the human oral cavity and is a significant contributor to tooth decay. S. mutans is one of a few specialized organisms equipped with receptors that improve adhesion to the surface of teeth. Sucrose is used by S. mutans to produce a sticky, extracellular, dextran – based polysaccharide that allows them to cohere to each other and form biofilm.[6]

Escherichia coli (E. COLI)

Escherichia coli is a Gram negative lactose fermenting rod – shaped bacterium that is commonly found in the lower intestine (colon) as a part of normal flora of the gut. Uropathogenic Escherichia coli, the most common cause of CAUTI Catheter – associated urinary tract infection form biofilms on indwelling catheters.

Camellia sinensis (Green Tea)

Camellia sinensis is the tea plant belongs to family Theaceae. A number of polyphenols have been isolated. Of that, catechins of flavanol group are very important from biological point of view. They constitute up to 30% of the dry leaf weight. The most com-

mon Green tea Catechins are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC).[7] Green tea is known to have potential of antibacterial activity. It is generally accepted that activity of tea polyphenols are better against gram positive bacteria than gram negative. In a study carried out in India, green tea extract was tested against many organisms including *Staphylococcus aureus, Vibrio cholera, Escherichia coli, Shigella* spp., *Salmonella* spp., *Bacillis* spp., *Klebsiella* spp. and *Pseudomonas aeruginosa.* The spectrum of activity was the same.[8-10] Other biological activities include anti-oxident, anti-angiogenic, neuroprotective and anti-cancer.[11-14]

Juglans regia (Dandasa)

Walnut is a common temperate forest tree found throughout the world. The plant belongs to the family Juglandaceae. The dried bark of *Juglans regia* (Dandasa) is locally available in Pakistan. This bark is used to improve oral hygiene by tradition. There are very few reports stating about side effects after their oral use but none is reported any severe toxicity outcomes.[15] It also has been used for eczema, prutitus, blisters and as blood cleanser and laxative.[16] The tree si rish in flavanoides include catechins, myricetin, another compound naphthohydroguinone and Vitamin C.[17] Different other bioactivities have also been previously reported including antiaging, antiproliferative, antimutagenic, and inflammatory and antinociceptive activities.[18-20]

MATERIAL AND METHODS

Microscopic Examination

Oral biofilm samples were collected from 50 patients in different dental clinics while 50 contaminated urinary catheter were collected from different hospitals of Karachi.

To check the purity of culture and to observe their morphological characteristics the isolates were observed by grams stain and biochemical tests. All clinical isolates were identified using immunology and Infectious Diseases Research Laboratory, Department of Microbiology, University of Karachi by standard biochemical methods.

Collection and Preparation of Natural Compounds

Plant Collection

Dried leaves of Camelliasinensis (green tea) and Juglans ragia (dandasa) is the dried barck of Persian walnut tree which is purchased from local market.

Preparation of Aqueous Extracts

A solution of each dried plant material was prepared in sterile distilled water by taking 5 mg/100 ml and heating at 95°C in water bath for two minutes and cooling for two minutes. Procedure was repeated three times and final extractions were centrifuged. Supernatant was filtered through 0.2 µm membrane stored at –20°C and thawed before used. Every time stored extract were used for not more than one week for different bioassays. 50 mg / ml of these products were used as initial concentration then further dilution was made accordingly.

Biofilm Forming Assay Through Elisa Reader

We performed two methods using 96 well plates for determining the biofilm forming ability of all cariogenic bacterial isolates.

A) In order to study the biofilm formation, culture was grown in Tryptone Soya Broth, matched with 0.5 McFarland and culture was transferred in each well of microtitre plate. Along with the test, controls were also run having *strep mutan,* E.coli, uninoculated broth and empty wells. Plates were made in duplicate, incubated and covered at 37°C for 24 h and 72 h. Cell turbidity was monitored using a microtitre plate reader at an optical density at 405 nm. After incubation medium was removed from wells and microtitre plate wells were washed with PBS to remove loosely associated cells, each well was stained with 100 µl of 1% crystal violet solution for 45 min and further washed 3 times with PBS over which 10% alcohol was added and O.D was recorded by. Measuring the absorbance using ELISA reader similarly another plate which was incubated for 72 hrs was read for O.D determination.

Bacterial Adhesion Assay

A 20 µl of pre-culture suspension match with McFarland 0.5 inoculated in glass tube containing 1 ml of Brain Heart Infusion broth plus 1 ml of tested compound of required concentration and then left this tube at an angle of 30 undisturbed for 18 hr at 37°C for culturing and adhesion. After the suspension transfer into new culture tube (fraction A), add 1 ml Brain Heart Infusion broth and 1 ml compound tested in empty tube from which we put in Fraction A then do vortexing for 30 sec then shift this to another culture tube (fraction B). Finally 1 ml of Brain Heart Infusion broth and 1 ml of tested compound add in the tube from which we put suspension in fraction B then sonication done so that bacteria which is tightly adhered detached and name this tube as (fraction C). Then check turbidity of each fraction at OD 550 nm adjusted tube having 1 ml Brain Heart Infusion broth and 1 ml compound only as OD 550 nm as 0. Then calculate the % adhesion by putting values in following formula (C / A + B + C)* 100,

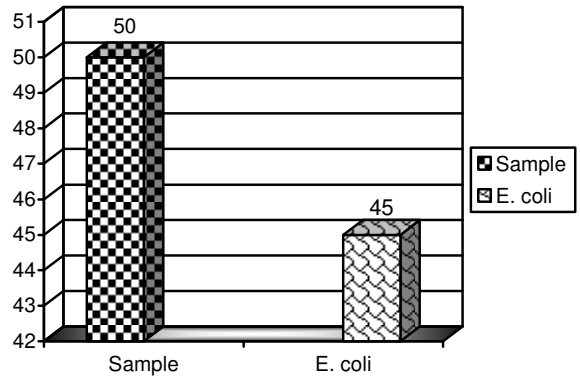
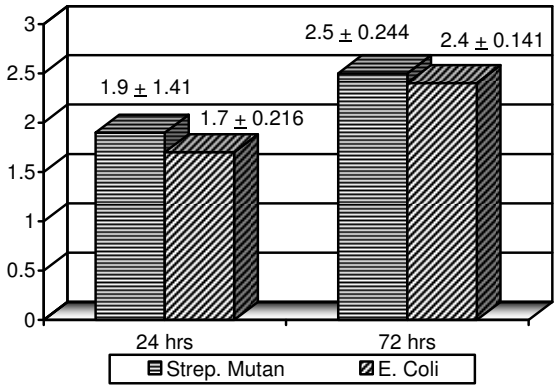


Fig. 1: *E. coli sample.*



Cutoff value > 1.0 biofilm former

Fig. 3: *Biofilm formation in 24 hrs and 72 hrs.*

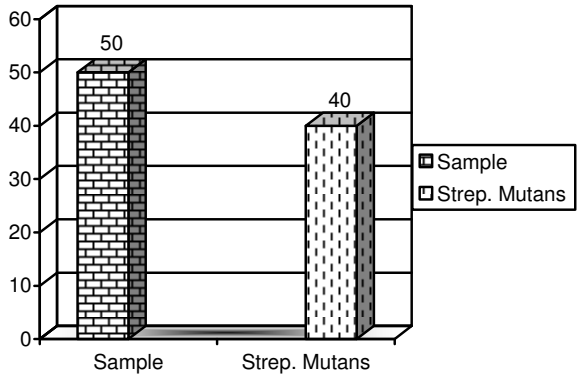


Fig. 2: *Streptococcus mutans sample.*

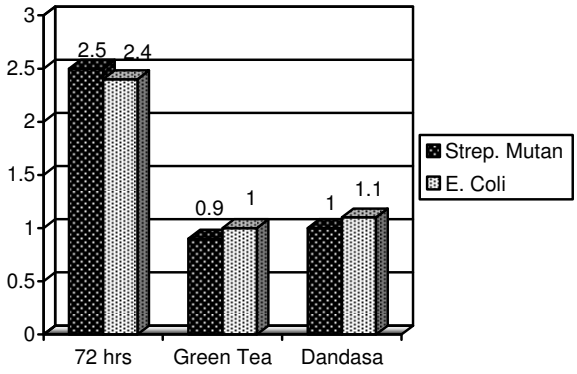


Fig. 4: *Biofilm in the presence of green tea and dandasa.*

WHERE A, B, C considered as turbidity of fractions at OD 550 nm.[21]

RESULTS

All test results will be assess through SPSS version and conducted in triplicate.

DISCUSSION

Now a days due to our busy schedule we ignore oral hyiegene not knowing the impact of this ignorance and on our health. As a result of this ignorance bad oral hyiegene, the bacteria from oral biofilm which provide protection to these bacteria there is a high incidence of cariogenic infection and their complication including cardiovascular diseases, tooth decay gingivitis etc due to the presence of microorganisms.

Table 1: *Anti-adhesive activity against E. coli.*

E. Coli				
Dandasa	*A*	*B*	*C*	
12.5 mg/ml	2.0 ± 0.244	1 ± 0.141	.5 ± 0.141	12.5% (effective)
6.2 mg/ml	2.5	1.5	1.5	25%

Green Tea	*A*	*B*	*C*	
3.2 mg/ml	2.0 ± 0.141	1.6 ± 0.141	.5 ± 0.141	12.1% (effective)
1.6 mg/ml	2.5	2.5	2.2	28.5%

In our study we analysed the antimicrobial and anti-biofilm activity of natural compounds like dandasa, and green tea. It is observed from the above results that there is 80% prevalence of streptococcus mutans in dental plaque sample as shown in Fig. 2 and there is 90% prevalence of E. coli in contaminated urinary catheter as shown in Fig. 1. Above results show that isolated organisms from dental plaque and urinary catheter form firm invitro bio-

film after 72 hr as in Fig. 3 but when we were using dandasa and green tea as anti-biofilm former then there is no biofilm formed even after 72 hrs as shown in Fig. 4. It is also observe in our study that anti-adhesive activity of dandasa is more effective as it shows 12% effectiveness even at 3.2 mg/dl concentration as compare to 12.5 mg/dl concentration of green tea gives the same results as shown in table 1. Whereas antiadhesive activity of green tea is more effective in case

Table 2: *Anti-adhesive activity against streptoco-ccus mutans.*

Streptococcus Mutans				
Dandasa	*A*	*B*	*C*	
6.2 mg/ml	2.0 ± 0.244	1 ± 0.141	.5 ± 0.141	12.5% (effective)
3.1 mg/ml	2.5	1.5	1.5	25%

Green Tea	*A*	*B*	*C*	
12.5 mg/ml	2.0 ± 0.141	1.6 ± 0.141	.5 ± 0.141	12.1% (effective)
6.2 mg/ml	2.5	2.5	2.2	28.5%

Table 3: *Combined effect of green tea and dandasa.*

	Green tea (effective)	*Combination*	*Dandasa (effective)*	*Combination*	*Antiadhesive Index*	*Relation*
E. Coli	3.2 mg/ml	1.6 mg/ml	12.5 mg/ml	3.2 mg/ml	0.756	Indifferent
Strep. Mutan	12.5 mg/ml	12.5 mg/ml	6.2 mg/ml	3.2 mg/ml	1.5	Indifferent

of E. coli as shown in table 2. Later in our study it has been observed that when we combine both these natural products then none of the compounding (green tea and dandasa) show synergistic or antagonist activity and both compound shows indifferent activity as shown in table 3. Therefore from the above mentioned results it was shown that green tea and dandasa were more effective when use separately although green tea was more effective in anti-biofilm activity against E. coli whereas dandasa having good activity against streptococcus mutans. Our study results were also similar to the previous studies on natural compound antibiofilm forming activity.[22]

therefore it is *concluded* from the above observation that it takes 3 days for an organism to develop proper biofilm and before that it is the time when organism usually form their attachment with that of surface over which they form biofilm and it is the right time when antibacterial or antibiofilm agent may be applied to prevent the formation because once biofilm forms it provide shelter to the micro-organism against antibacterial agents. We have to remove biofilm using mechanical methods. It is also concluded that natural product like green tea and dandasa play a vital role in prevention of these biofilm with no side effect as compare to conventional antibiotics.

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