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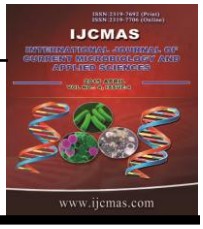

Original Research Article

Effect of aqueous extracts of different medicinal plants on control of *Streptococcus mutans*

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A B S T R A C T

Effect of aqueous extracts of different medicinal plants on control of *Streptococcus mutans* under in *vitro* condition was studied at MGM College of Agricultural Biotechnology, Aurangabad. The experiment was laid out in completely randomized design with twelve treatments of aqueous extracts of different medicinal plants (*Xanthocarpum solanum*, *Azadiracta indica*, *Osimum sanctum*, *Cinnamomum verum*, *Accacia nilotica*, *Foeniculum vulgare*, *Psidium guajava*, *Aloe vera*, *Elettaria cardamomum*, *Syzygium aromaticum*) with positive and negative control against pure culture of *Streptococcus mutans.* Among, *Syzygium aromaticum* showed significantly higher zone of inhibition on the Mutans-Sanguis agar plates in agar well diffusion assay. While on the broth culture *Syzygium aromaticum* and *Psidium guajava* showed inhibition of biofilm formation significantly in anti-biofilm activity. On an average, the present finding indicate that aqueous extracts of *Syzygium aromaticum* exert inhibitory effect on cariogenic properties of *S. mutans* and can be used to cure dental problems.

Keywords

Cotton, Ovule culture, IAA, NAA and Fiber length and weight

Introduction

Streptococcus mutans is a facultatively anaerobic, Gram-positive coccus-shaped bacterium commonly found in the human oral cavity and is a significant contributor to tooth decay. *Streptococcus* is a genus of spherical Gram-positive bacteria belonging to the phylum Firmicutes and the lactic acid bacteria group. Conditions in the oral cavity are diverse and complex, frequently changing from one extreme to another. Thus, to survive in the oral cavity, *S. mutans* must tolerate rapidly harsh environmental fluctuations and exposure to various anti-microbial agents in order to survive (Biswas S. and Biswas, I. 2011). *S. mutans* is more prevalent on the pits and fissures, constituting 39% of the total *streptococci* in the oral cavity while fewer *S. mutans* are found on the buccal surface (2-9%). (Ikeda T. and Sandham H., 1971).

S. mutans was first identified by Kilian Clarke who was a microbiologist (Clarke, 1924). One of its important virulence properties is the ability to form biofilms (dental plaque) on tooth surfaces.

Eradication of such biofilms is extremely difficult agent of human dental caries. (Kunze *et al*, 2010) The break-down of lactic acid formed due to high levels of sugar are consumed, proceeds much more rapidly compared with other bacteria (Hamada and Slade 1980). The metabolism takes place in both neutral and acid environments, with continuing activity at low pH values (Köhler *et al*. 1995).

Extracellular polysaccharides are also produced during the course of enzymatic reactions. Their stickiness favours the adherence of bacteria on tooth surfaces, which enables them to settle on even very smooth surfaces (Koga *et al*. 1986). Given their numerous reception sites for microorganisms, polysaccharides also promote the cross linkage and multiplication of plaque. Moreover, their insolubility in water hampers the natural protective effect of saliva. Intracellular polysaccharides ensure bacterial survival during low-nutrition intervals, and are used by the bacteria to produce further acids (Hamada and Slade 1980). They trigger the process that leads to initial mineral loss and that enables bacteria to penetrate the tooth structure (Burne 1997).

It has been well documented that traditional medicinal plants confer considerable antibacterial activity against various micro-organisms. Here, the experiment was conducted with the variety of medicinal plant extracts such as Kantkari (*Xanthocarpum solanum*), Neem (*Azadirac*ta *indica*), Basil (*Osimum sanctum*), Cinnamon (*Cinnamomum verum*), Babool (*Accacia nilotica*), Fennel seed (*Foeniculum vulgare*), Guava (*Psidium guajava*), Korphad (*Aloe vera*), Cardamom (*Elettaria cardamomum*), Clove (*Syzygium aromaticum*). (Kumar D. and Sidhu P., 2003)

Neem (*Azardirachta indica)* is referred to as the village pharmacy in India because of its ability to cure many disorders ranging from bad teeth and bed bugs to ulcers and malaria. Neem is of particular interest to the field of dentistry for it has a long history treating teeth and gum problems. Cinnamom (*Cinnamomum verum*) is used internally as Cassia oil contains 80- 90 % cinnamaldehyde, used mainly in medicine, foods and cosmetics. Various parts such as the fruits, oil, inner bark and leafy twigs of cinnamon are used. The inner bark is a pungent, sweet, hot herb that treats many diseases. Babool (*Accacia nilotica*) which has long been used for the treatment of skin, sexual, stomach and tooth problems .It has been proved as effective medicine in treatment of malaria, sore throat (aerial part) and toothache. Also, seeds of Kantkari (*Solanum Xanthocarpum*) were used to prepare extracts to check antimicrobial activity. (Kumar D. and Sidhu P., 2003)

Korphad (*Aloe vera*) is a medicinal plant with anti-inflammatory, antimicrobial, antidiabetic and immune-boosting properties. The mucilaginous tissue has traditionally been used for treatment of digestive tract disorders, sunburn and wounds. The pharmacological actions of Aloe vera gel as studied *in vitro* and *in vivo* include anti-inflammatory, antibacterial, antioxidant, immune-boosting and hypo-glycemic properties (Fani M. and Kohanteb J., 2011)

Tulsi (*Ocimum sanctum*) leaves are quite effective for the ulcer and infections in the mouth. It is also useful in pyorrhea and other gum disorders. The anti-inflammatory and anti-infectious properties of tulsi make it a powerful treatment for gum disease. In southern Nigeria the twigs of guava (*Psidium guajava*) are used as chew sticks and the presence of bioactive compounds

comprised of saponins, tannins, flavonoids, alkaloids is responsible for their effectiveness. (Kukreja B. and Dodwal V., 2012)

Clove (*Sygium aromaticum*) is a dried, unopened inflorescence of clove tree, which contains 20%essential oil. Cloves are highly pungent due to presence of eugenol which is reported to have strong anti-fungal and anti-inflammatory activities, and has been used in dentistry as an abundant. It can be produced by distillation to yield essential oil. Cloves' buds have been regarded as safe when taken orally to disinfect root canals, temporary fillings and as an oral anesthetic. Eugenol is a chemical compound present in cloves and is known to inhibit growth of bacteria. It is a natural antibiotic with broad antimicrobial activities against gram-positive, gram-negative, acid-fast bacteria, as well as fungi. (Rahim Z., 2005)

To solve the problem of dental caries and find out a substance which can be used to reduce or eradicate *Streptococcus mutans* with least amount of side effects, the research work entitled "Effect of aqueous extracts of different medicinal plants on control of *Streptococcus mutans*" has been planned with following objectives. To Formulate aqueous extracts from selected plant. To Study the effect of medicinal plant extract on inhibition of *S. mutans*. To Study the biofilm inhibition ability of medicinal plant aqueous extract.

Materials and Methods

Procurement of plant material

The fresh plant material of Kantkari (*Solanum xanthocarpum*), Neem (*Azadiracta indica*), Basil (*Osimum sanctum*), Cinnamon (*Cinnamomum verum*), Babool (*Accacia nilotica*), Fennel seed (*Foeniculum vulgare*), Guava (*Psidium guajava*), Korphad (*Aloe vera*), Cardamon (*Elettaria cardamomum*), Clove (*Sygium aromaticum*) were collected from the local market and field. (Mohammed N., 2012).

Table 3.1:- List of plant material selected for study

Treatment	Aqueous extracts
T_0	Distilled water
T_1	Absolute ethanol
T_2	Aqueous seed extract of *Solanum Xanthocarpum*
T_3	Aqueous seed extract of *Elettaria cardamomum*
T_4	Aqueous seed extract of *Foeniculum vulgare*
T_5	Aqueous bark extract of *Cinnamomum verum*
T_6	Aqueous bud extract of *Syzygium aromaticum*
T_7	Aqueous leaf extract of *Osimum sanctum*
T_8	Aqueous leaf extract of *Aloe vera*
T_9	Aqueous twig extract of *Azardirachta indica*
T_{10}	Aqueous twig extract of *Psidium guajava*
T_{11}	Aqueous twig extract of *Accacia nilotica*

Preparation of aqueous extracts:

The plant material was dried in shade for 3-4 days and powdered with the help of grinder. 10gm powder of each plant material was mixed with 100ml distilled water. The mixture was then heated slowly to 90˚C for an hour & filtered through several layers of Whatman filter paper. This filtrate was concentrated to 1/5 of the original volume by evaporation in shaded condition and stored at 4˚C. (Mohammed N., 2012)

Media Preparation

Preparation of MSB agar medium:

The MSB Agar (mitis-salivarius-bacitracin) medium is usually used for the isolation of *Streptococcus mutans*. Although it is considered as a selective culture medium for this micro-organism, *S. mutans* recovery in this medium is much lower than Mitis Salivarius agar. Because the number of *S. mutans* in saliva is used for estimating caries risk and activity from a microbiological stand point. (Annan *et al*, 1997)

Table.3.2 Mitis-Salivarius-Bacitracin Agar

Sr. no.	Constituents	Amount (gm/lit)
1	Sucrose	50
2	Enzymatic digest of protein	10
3	Proteose Peptone	10
4	Dipotassium Phosphate	4
5	Dextrose	1
6	Trypan Blue	0.08
7	Crystal Violet	0.80
8	Agar	15
9	Potassium Tellurite (1%)	10ml
10	Bacitracin	2,00,000 units
11	pH (at 25^{o} C)	7.0

(Atlas R., 1997)

The above concentration was mixed with distilled water except K-tellurite and bacitracin, plugged well and autoclaved the medium at 121^{0}C (15 psi) for 15 min. K-tellurite and bacitracin were added to the medium after lowering the temperature to 45-55^{0}C and mixed well.

Preparation of Mutans-Sanguis agar medium

For the differentiation of *S.mutans* and *S. sanguis* strains of mutans group, MS agar was used. It was found easy to grow and handle the pure culture of *S.mutans* for further activity (Atlas R., 1997).

The given concentration was mixed with distilled water and autoclaved the medium at 121^{0}C (15 psi) for 15 min. In this case K-tellurite and bacitracin were not added to the medium to achieve stress less growth of bacteria on the plates.

Table.3.3 Mutans-Sanguis-Agar

Sr. no.	Constituents	Amount (gm/lit)
1	Casein Enzymic Hydrolysate	15
2	Yeast Extract	5
3	L-Cystine	0.200
4	Sodium Sulphite	0.100
5	Sodium Chloride	1
6	Disodium Phosphate	0.800
7	Sodium Bicarbonate	2
8	Sodium Acetate	12
9	Sucrose	50
10	Agar	15
11	pH (at 25^{o} C)	7.3

(Atlas R., 1997)

Isolation of pure culture

The bacteria firstly isolated from saliva in saline (0.87% NaCl) and cultured on nutrient broth at 37°C for 18-24 h. Secondly, MSB medium was sub-cultured with white coloured sticky colonies from nutrient agar plates and incubated at 37ºC for 24 h. from which *S. mutans* was identified by different biochemical tests and its morphology by Grams staining method (Najah M., 2012). *S. mutans* culture was prepared on MS agar medium in test tubes and kept as pure culture at refrigerator for further activity.

Biochemical Tests

Table.3.4 Biochemical tests for *Streptococcus mutans*.

Tests	Operating instruction	Probable Results
Gram Staining	Prepare a smear on a clean slide. Take few drops of crystal violet, wait for 1 min. and wash with d/w. Flood with Gram's iodine for 1 min. Wash with Gram's decolourizer till iodine colour disappears. Then take 2-3 drops of safranin and wash with d/w after 1 min.	Violet coloured colonies indicate *S.mutans* as it is a Gram positive bacteria. Check purity.
Motility Test	Stab with a needle straight in and straight out of the center of the tube half way down. Incubate for 24 hours at 37°C in CO_2	Motile organisms have obvious growth away from inoculation area; Non-motile organisms grow only in inoculation area
Catalase Test	Transfer a well-isolated colony to a clean slide & add 1 drop of 3% H_2O_2. Do not reverse the order & do not mix. Observe for immediate bubble formation.	Bubble formation should occur when test is positive. *S. mutans* is catalase negative.
Mannitol Test (Carbohydrate Fermentation Test)	Prepare Mannitol Salt Agar and spread the colonies over it.	Mannitol utilizing organisms turn the red medium to yellow.
Resistance	Add bacitracin to the media as *S.mutans* shows resistance to the antibiotic.	All types of bacteria are inhibited other than *S.mutans.*

(Whiley and Beighton, 1998)

Antibacterial Assay

Antibacterial activity of the extracts was determined using the agar well diffusion assay method (Perez et al., 1990). Overnight grown culture of *Streptococcus mutans* was spread on the agar surface using sterile spreader. Agar wells of 0.5cm diameter were prepared with the help of stainless steel cork borer. The wells in each plate were loaded with 50 µl of aqueous extracts of selected medicinal plants. Distilled water and ethanol were kept as negative and positive controls respectively. The plates were incubated at for 37^0C 24 hr. All the tests were repeated in triplicates. The antibacterial activity is noted on the basis of diameter of zone of inhibition with the help of zonal scale, which was measured at cross-angles after 24 hr of incubation (Kumar D. and Sidhu P., 2003).

Antibiofilm Assay

A qualitative assessment of biofilm formation was determined by tube method. Mutans-Sanguis broth supplemented with sucrose was inoculated with loopful of microorganism from overnight culture plates and desired plants extract in different test tubes and then incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film on wall and bottom of the tube. Experiments were performed in triplicate. (Pieri et al, 2012).

Results and Discussion

Biochemical Tests

The results of bacterial identification were observed by using the microscopical examination and biochemical tests, where in the microscopical examination the colonies of *Streptococcus mutans* were found spherical, positive gram staining cocci, arranged in pairs, having short chains with capsule, non- spore forming and non-motile.

Effect of different aqueous plant extracts on growth of *S.mutans*

The effect of aqueous extracts of *Solanum xanthocarpum, Elettaria cardamomum, Foeniculum vulgare, Cinnamomum verum, Sygium aromaticum, Osimum sanctum, Aloe vera, Azadiracta indica, Psidium guajava* and *Accacia nilotica* on *Streptococcus mutans* is tabulated in table 4.2.

Data presented in Table 4.2 revealed that zone of inhibition of *S.mutans* was influenced significantly due to different aqueous extracts of medicinal plants. *Sygium aromaticum* resulted widest zone of inhibition for *S. mutans* (29 mm) and was found significantly superior over rest of the treatments. *Psidium guajava* (15mm), absolute ethanol (14.33mm), *Accacia nilotica* (12.33mm), *Solanum xanthocarpum* (11.33mm) and *Cinnamomum verum* (11.33mm) did not differ significantly and recorded higher zone of inhibition over *Azadiracta indica, Osimum sanctum, Elettaria cardamomum, Foeniculum vulgare, Aloe vera* and the negative control. It is interesting to note that lower antimicrobial activity was seen in Neem extract which is traditionally used to keep oral hygiene. But, *Azadiracta indica* was observed at par with *Psidium guajava*, absolute ethanol, *Accacia nilotica*, *Solanum xanthocarpum* and *Cinnamomum verum* thus can be measured as significant treatment. However, remaining medicinal plant extracts, such as *Osimum sanctum, Elettaria cardamomum, Foeniculum vulgare, Aloe vera* and the negative control i.e. D/W were at par with each other in inhibition of *S.mutans* with the least zone of 1, 0.9, 0.8, 0.8, 0.4 respectively and proved significantly inferior as compare to rest of the treatments in regarding clear zone. *Sygium aromaticum* was found effective in inhibition of *S. mutans* as it contain eugenol which is an antimicrobial agent.

Table 4.1 Result of Biochemical test of *S. mutans*

Sr. no.	Tests	Observations	Result
1	Gram's Staining	Violet coloured cocci, arranged in pair, short to medium chains with capsules.	+
2	Motility Test	Non-motile	_
3	Catalase Test	Bubble formation did not occur.	_
4	Mannitol Test (Carbohydrate FermentationTest)	Colour change was observed from red to yellow.	+
5	Resistance (Bacitracin)	*S.mutans* showed resistance to the antibiotic used and formed an even lawn on the agar plate.	+

Antibiofilm Assay

Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong. Data presented in Table 4.3 indicated that positive and negative control had performed significant role of eradication of biofilm formed due to growth of *S.mutans.* Here,

again *Sygium aromaticum* showed best inhibitory effect over control of biofilm when compared to absolute ethanol followed by *Psidium guajava* and *Accacia nilotica.* Strong biofilms were observed on the tubes having plant extracts of *Osimum sanctum, Aloe vera* and *Elettaria cardamomum* while *Foeniculum vulgare* can be concluded as moderate one.

Present investigation entitled "Effect of aqueous extracts of different medicinal plants on control of *Streptococcus mutans*" was carried out *in vitro* conditions during Nov 2012 to April 2013 in Biochemistry and Molecular Biology Laboratory of MGM College of Agricultural Biotechnology, Aurangabad. Experiment was laid out in Completely Randomized Design with twelve treatments (positive control, negative control and medicinal plant extracts with same volume) and three replications.

Table.4.2 Effect of different aqueous plant extracts on growth of *S.mutans*

Treatment no.	Treatment (Plant extract)	Zone of inhibition (in mm)
T_0	Control (D/W)	00.40
T_1	Control (absolute ethanol)	14.33
T_2	*Solanum xanthocarpum*	11.33
T_3	*Elettaria cardamomum*	00.90
T_4	*Foeniculum vulgare*	00.70
T_5	*Cinnamomum verum*	11.33
T_6	*Sygium aromaticum*	29.00
T_7	*Osimum sanctum*	01.00
T_8	*Aloe vera*	00.70
T_9	*Azadiracta indica*	09.33
T_{10}	*Psidium guajava*	15.00
T_{11}	*Accacia nilotica*	12.33

S.E. = 1.382, C.D. =4.165

Aqueous extracts of different medicinal plants were prepared for the antibacterial activity and stored at 4ºC until used. A pure culture of *Streptococcus mutans* was prepared in the lab using sterile MSB agar and MS agar respectively and confirmed as *S.mutans* after different biochemical tests. Bacterial culture was maintained at 20˚C and recultured after every week.

The agar well diffusion assay method included punching of agar with the help of stainless steel cork borer of about 5mm in diameter. 50µl aqueous extracts were loaded within it and zone of inhibition was measured of each replication after 24 hrs of inoculation. Similarly, a qualitative assessment of biofilm formation was determined by tube method. MS broth was inoculated with loopful of microorganism and 1ml plants extract. The tubes were washed with PBS followed by crystal violet staining. Biofilm formation was considered positive when a visible film on wall and bottom of the tube was observed.

Present study is in agreement with Kumar D. and Sidhu P. who reported that clove extracts produce antibacterial activity against gram positive strains and multiple antibiotic resistant bacteria as clove showed most pronounced antibacterial activity as compared to control and can be used for maintaining oral hygiene.

This in- vitro study gives us natural antimicrobial agents which can help us to control dental caries and endodontic infections and the data suggests that the herbal can provide oral health benefits by inhibiting the growth of cariogenic oral pathogen.

Table.4.3 Antibiofilm Assay

Treatment no.	Treatment (Plant extract)	Replication no.	Biofilm formation			
			0	1	2	3
T_0	Control(D/W)	1				√
		2				√
		3				√
T_1	Control(absolute ethanol)	1	√			
		2	√			
		3	√			
T_2	*S. xanthocarpum*	1			√	
		2			√	
		3			√	
T_3	*Elettaria cardamomum*	1				√
		2				√
		3				√
T_4	*Foeniculum vulgare*	1			√	
		2			√	
		3			√	
T_5	*Cinnamomum verum*	1			√	
		2			√	
		3			√	
T_6	*Sygium aromaticum*	1	√			
		2	√			
		3	√			
T_7	*Osimum sanctum*	1				√
		2				√
		3				√
T_8	*Aloe vera*	1				√
		2				√
		3				√
T_9	*Azadiracta indica*	1			√	
		2			√	
		3			√	
T_{10}	*Psidium guajava*	1		√		
		2		√		
		3		√		
T_{11}	*Accacia nilotica*	1		√		
		2		√		
		3		√		

- Clove extracts have the potential to inhibit the plaque inducing properties of *S. mutans* by affecting cell adhesion and glucosyltransferase activities which subsequently affect the caries inducing properties of bacteria and can help to reduce root canal microflora.
- Guava and Babool extracts should be used in oral health care products as their degree of effectiveness differs within a narrow range, making the two extracts equally effective to inhibit the growth of cariogenic pathogens without any side effect.

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