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To study the biofilm formation and antibacterial activity of some medicinal plants against *Streptococcus* sp. isolated from diabetes type 2 patients

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ABSTRACT

Type 2 diabetes patients have significantly higher plaque index and a higher prevalence and magnitude of root surface caries than non-diabetic subjects. In the present study we have isolated two *Streptococcus* sp. from oral samples of diabetes type 2 patients and studied the biofilm formation by these bacteria. Biofilm formation act as a defensive method during various stress conditions for some bacteria. *Streptococcus mutans*, the principle cariogen for dental caries, co- exist with over 500 other species of bacteria as an interactive community known as the dental biofilm (Jubai 2015). The inhibitory effect of some medicinal plants against the isolated *Streptococcus* sp. were also studied. For this purpose Walnut (*Juglans regia)* stem bark, cinnamon (*Cinnamomum zeylanicum)* and clove (*Syzygium aromaticum)* extracts were used. The results obtained displayed that the aqueous extract of clove exhibits highest antibacterial activity against these isolated biofilm forming *Streptococcus* sp. C1 and *Streptococcus* sp. C2.

INTRODUCTION

Type 2 diabetes, non insulin dependent diabetes mellitus (NIDDM), or adult-onset diabetes, is characterized by the progressive degeneration of islet β-cells of the pancreas resulting in a fall of insulin secretion and decreased insulin action on peripheral tissues. The relationship among diabetes mellitus, periodontal health and subsequent tooth loss have been broadly reported (Campus et al., 2005; Khader et al., 2006; Furukawa et al., 2007; Taylor and Borgnakke, 2008). There is ample evidence of the biological and epidemiological links between periodontal disease and diabetes, especially, type 2 diabetes mellitus. The high percentage human infections result from facts that micro-organisms present in the biofilm are highly resistant to killing and to treatment with microbial agents. Bacteria forms biofilms largely to remain in a favourable niche (Jefferson, 1994). The bacteria constituting the oral biofilm varies at distinct sites as a result of the inherent biological and physical properties at these sites. Plants have a great potential for producing new drugs of great benefit to mankind. There has been a considerable interest to use plants and spices for the elimination of microorganisms because of increasing antibiotic resistance of microorganisms (Kunin, 1993; Finch, 1998; Smid and Gorris, 1999). The aim of the present study was to isolate biofilm forming bacteria from the oral samples of diabetes type 2 patients and to investigate the antibacterial activity of some medicinal plants (Walnut (*Juglans regia*) stem bark, cinnamon (*Cinnamomum zeylanicum*) and clove (S*yzygium aromaticum*) against the isolated bacteria. The results obtained could be useful for the development of new tools as antimicrobial agents for the control of oral diseases in diabetes type 2 patients.

MATERIALS AND METHODS

Sample collection of oral bacteria

Twelve different samples were collected from oral of diabetic type 2 patients by swabbing across the dental caries. The samples were collected from Minocha Diabetic Centre, Dehradun. The collected samples were inoculated in Nutrient broth (HiMedia, India) and viable cells were enumerated.

Microbiological and biochemical characteristics of isolated bacteria

Gram staining was performed to observe the cellular morphology and gram nature of the isolated bacteria. Biochemical characterization of the isolated bacteria were also carried out. The biochemical tests for sugar utilization; amino acid decarboxylation; catalase and oxidase production; nitrate reduction; hydrogen sulfide production and starch hydrolysis were performed (Pacarynuk and Danyk, 2004; Collins et al., 1989).

Screening of biofilm forming bacteria by tube assay

A qualitative assessment of biofilm formation was determined as previously described by Christensen et al. (1982). Here, one loopful from the subcultures of both isolates were inoculated in 5ml LB broth containing test tubes and incubated for 4 h at 37˚C for enrichment. Then the suspension were dispensed in different test tube at inoculum to medium ratio of 1:20 and incubated at 37˚C temperature for different periods (24, 48, 72, 96 and 120 hours respectively). After incubation, biofilm was seen by staining procedure. The dye used for this purpose was ammonium oxalate crystal violet. In this assay, after the respective incubation times, the culture medium was discarded from the tubes carefully. Then the tubes washed with sterile distilled water to remove loosely associated bacteria and air dried for 30 min. The tubes were stained with 1% ammonium oxalate crystal violet solution and left for 30 min at room temperature. Tubes were then inverted to remove the crystal violet and rinsed twice with sterile distilled water to remove excess crystal violet (CV). The test was considered positive when there was a layer of stained material on the inner surface of the tube. Adherence was estimated as absent, weak, moderate or strong.

Microtitre plate assay

The quantitative estimation of the biofilm formation was done by Microtiter plate assay. The isolates were grown in trypticase soy broth (TSB) with 5% sucrose and incubated overnight at static condition at 37°C to obtain sufficient bacterial growth. The cultures after 24 hours were diluted 100 times with the same medium and 200 µl of the culture were inoculated in the 96 well plate in triplicates. The 96 well plate was incubated for 24 hours in static condition at 37°C at static condition. After respective incubation period content of each well was gently removed by slightly tapping the plates. The wells were then washed with phosphate buffer saline (PBS pH 7.3) to remove free-floating planktonic bacteria. The plates were then stained with 0.1% (w/v) crystal violet solution. Excess stain was washed off thoroughly with 95% ethanol and plates were kept for drying. Optical density (OD) was measured using micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of attachment to surface. The experiment was performed in triplicates and average reading was considered.

Stress response Study

The isolated strains were studied for biofilm formation under various stress conditions like sugar, salinity and pH.

Sugar stress

Stock solution of five sugars, i.e.; glucose, sucrose, arabinose, fructose and xylose having concentration of 10x was prepared in autoclaved water and sterilized by filtering through 0.22µm pore size membrane filters and stored at 4ºC. 900 µl of media (containing respective sugar in 1% conc.) was inoculated with 100 µl of overnight broth culture of the bacterial strains. 100 µl of the same concentration of inoculum was inoculated in 96 well microtitre plates and was incubated at 37ºC for 72 hours. After incubation, the contents of the plates were removed by tapping and was rinsed with phosphate buffer saline (PBS). The attached biofilm was stained with 10 times diluted grams crystal violet for 15 minutes followed by destaining with 95% ethyl alcohol. The alcohol was rinsed and allowed to dry. The O.D. of

the adherent biofilm was taken using a micro ELISA auto reader at 570nm.The tubes were stained in similar manner and results obtained with different sugars were compared.

Salinity

Salt stock of concentration 1mg/ml was prepared in autoclaved water and filter sterilized using 0.22µm membrane filters. The salt stock was serially diluted in LB broth to obtain a concentration of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. 1 ml of media with respective salt concentration was inoculated with overnight culture in 1:10 dilution. 200 µl of same inoculum concentration was inoculated into 96 well microtitre plates and incubated at 37ºC for 72 hours. After incubation the tubes and plates were stained and analyzed as described above.

pH stress

To observe the effects of media pH on the biofilm formation by selected isolates, Luria Bertani (LB) media were prepared in buffer solution. After adding the ingredient of LB broth, the pH of the media was adjusted to the specific pH points to pH 3, 4, 5, 6, 7, 8 and 9. Then 5 ml of medium from each pH was transferred to 6 inch screw cape tubes. After autoclaving the tubes were inoculated with single organism, and incubated at optimized conditions for each isolates. After incubation the biofilm was assayed by the modified method. The pH effects on the biofilm formation were recorded.

Antimicrobial assay

Collection of plant material

We have selected three Indian medicinal plants for antimicrobial assay, based on their ethnomedicinal and traditional uses against infectious diseases based on literature survey and interaction with herbal healers. Plant stem barks of walnut were collected randomly from forest and plants buds of cinnamon and clove were collected randomly from local market. Whole plants bud of cinnamon and clove and whole plant stem bark of walnut was taken for investigation of antimicrobial property.

Extraction and antimicrobial assay

For aqueous extraction, 10 g of air-dried powder of plants materials were added to distilled water and boiled on slow heat for 2 h. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant collected at an interval of every 2 h, was pooled together and concentrated to make the final volume one-fourth of the original volume (Parekh et al., 2005).

For methanol and benzene extracts, 10 g of the dried and powdered plant materials were extracted with 100 ml of solvents by using soxhlet apparatus for 10 h at a temperature not exceeding the boiling point of the solvents (Lin et al., 1999). The obtained extracts were filtered by using whatman No. 1 filter paper and then concentrated under vacuum at 40 °C by using a rotary evaporator. The residual extracts were stored 4 0C for further use. The antimicrobial assay was performed by disc diffusion method (Lorian,1996).

Muller Hinton (MH) agar (HiMedia Lab) was used to prepare the culture medium and autoclaved at 121˚C for 15 min. Briefly, plates (8-cm diameter) were prepared with 1.9 ml MH agar inoculated with 0.1 ml of bacterial suspension suspension (0.1 ml of 0.5 Mc Farland Standard). The extracts were dissolved in their respective extracted solvent. Sterile paper discs (6 mm in diameter) were impregnated with 0.02 ml of 100 mg/ml concentration of extract placed onto MH agar. The plates were incubated at 37˚C overnight. Negative controls were prepared using the same solvent employed to dissolve the plant extract. Tetracycline (30 µg) antibiotic disc were prepared from Difco. were tested in the same conditions as positive controls used as positive controls. Inhibition zones in mm (without disc paper diameter) around discs were measured. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extract against test microorganisms. The experiment was repeated in triplicate and the mean of diameter of the inhibition zones was calculated.

Minimum Inhibitory Concentration (MIC)

Minimal Inhibitory Concentration Assay by Serial Dilution Method Minimum inhibitory concentrations (MICs) were determined by broth dilution method in culture tubes (Jorgensen et al 1999) with some modification. In the tube dilution assay, the extract was initially prepared at 50 mg/ml. Then standard bacterial suspension and different concentrations of extract (0.34, 0.68, 1.3, 2.56, 5 and 9.75) were added to tubes containing 1.9 ml Muller-Hinton Broth (HiMedia Lab). Each tube was inoculated with 0.1 ml of suspension containing 107 CFU/ml of each bacterium and incubated at 37˚C for 24 h. The negative control tube received no antimicrobial agent, and the positive control tube received no concentration of extract. The tubes were examined for visible growth or lack of growth for each dilution of test bacteria. Turbidity indicated growth of the microorganism.

Minimum Bactericidal Concentration Assay

The MBC values of the extract were determined by the drop plate method from the tubes, where apparently no visible growth found according to Kowser and Fatema 2009. Some modifications were made to the method. The Minimal Bactericidal Concentration (MBC) assay was performed as an adjunct to the MIC and was used to determine the concentration of extract was lethal to the target bacteria in vitro. From each MIC broth tube without visible growth, 25 µl volume of the broth was aliquot onto Nutrient agar and spread across the entire surface of the plate. Then the dilution of the sub cultured MIC tube was recorded on each plate and incubated at 25˚C for 24 h. The MBC plates were examined for colony growth or lack of growth for each dilution sub cultured. No growth indicated that the extract was bactericidal at that dilution. Growth indicated that the extract was bacteriostatic but not bactericidal at that dilution.

RESULTS AND DISCUSSION

Isolation and identification of dental caries causing bacteria

Fifteen bacterial isolates were obtained from the collected samples. Among these *Streptococcus* sp. (C1 and C2) were reported from 10 samples and the *Lactobacilli* sp. were isolated from 6 patients. HiMedia Rapid Biochemical Identification kit was used for the identification of bacteria based on biochemical characteristics. The Streptococcus sp. C1 and C2 were differentiated on the basis of biochemical characteristics, the detection of which aid in the identification and classification of microorganisms that appears morphologically identical (Table1). The results indicated that the *Streptococcus* sp. C1 ferments sugars glucose and sucrose both whereas *Streptococcus* sp. C2 ferments only glucose.

Biofilm formation by *Streptococcus* sp. C1 and C2

Streptococcus sp. C1 and C2 isolated from oral samples of diabetes type 2 patients were examined for biofilm formation by tube assay and microtitre plate assay. It was observed that biofilm was formed after 24h by these bacteria. Biofilm formation act as a defensive method during various stress conditions. Bacteria forms biofilms largely to remain in a favourable niche (Jefferson, 1994). Rachid et al. (2000) reported that oral bacterial species mostly lack an environmental niche and are found almost exclusively within the mouth (Fig 1).

Stress response study

Sugar stress

Biofilm formation was screened using five different sugars (glucose, sucrose, fructose, arabinose and xylose as the sole carbon source in basal media. Glucose was found to be good source of carbon as both the strains shows best attachment whereas sucrose was found to be good source in case of *Streptococcus* sp. C1 (Table 2).

Salt stress

Biofilm formation was studied under different conc. of salt (0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8 mg/ml and 1mg/ml) it was observed that it decreases with increase in salt concentration (Table 3).

pH Stress

Biofilm formation was studied under different pH conditions (6.4, 7.4 and 8.4). It was observed that in the range of pH 7.4 and 6.4 both the species displayed good biofilm attachment. Biofilm formation was poor at pH 8.4 (Table 4).

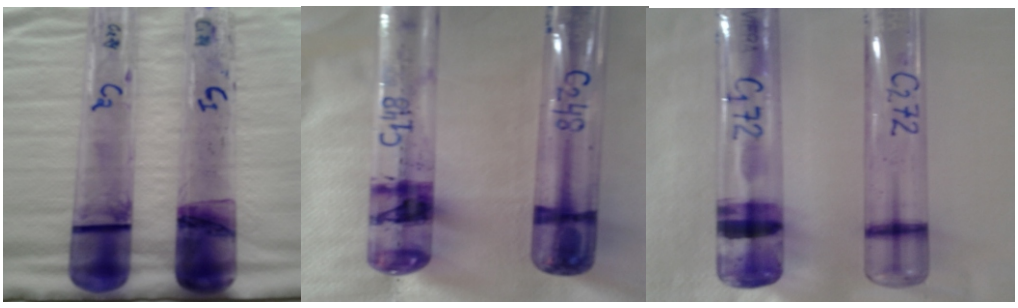

Figure 1: Biofilm production of *Streptococcus* sp. C1 and C2 after 24h, 48h and 72h

Table 1: Morphological and biochemical identification for the confirmation of the isolated microbes

S. No.	Characters	*Streptococcus* sp.	*Lactobacillus* sp.
1.	Gram staining	+	+
2.	Colony morphology	Small, blue in colour	Large, gummy colonies
3.	Cells morphology	Cocci in chains	Cocci in chains
4.	Catalase	-	-
5.	**Starch hydrolysis**	-	-
6.	**Growth anaerobically**	+	+
7.	**Oxidase**	-	-
8.	**Starch hydrolysis**	-	-
9.	Growth on special media:		
	On Mitis–salivarius agar	**Small blue colour colonies**	-
	On Rogosa agar	-	**Small white colour Colonies**
10.	**Esculin hydrolysis**	+	+
11.	**Hippurate hydrolysis**	-	-
12.	**Arginine hydrolysis**	-	-
13.	Bacitracin sensitivity test (0.1 unit)	-	-
14.	Optochin sensitivity test	-	-
15.	Fermentation test:		
	Lactose	+	+
	Sorbitol	+	–
	Ribose	-	-
	Inulin	+	-

Table 2: Effect of different sugars on Biofilm formation

Isolate	Glusoce (Optical Density)	Sucrose (Optical Density)	Arabinose (Optical Density)	Fructose (Optical Density)	Xylose (Optical Density)
Streptococcus sp. C1	0.540	0.510	0.076	0.380	0.100
Streptococcus sp. C2	0.520	0.190	0.062	0.072	0.053

Indication: Strong producer more than 0.240 nm; weak producer between 0.120-0.240 nm while non-producer less than 0.120 nm.

Table 3: Effect of salt concentration on biofilm formation

Isolate	**Salt concentration (**0.2 mg/ml) (Optical Density)	**Salt concentration (**0.4 mg/ml) (Optical Density)	**Salt concentration (**0.4 mg/ml) (Optical Density)	**Salt concentration (**0.8 mg/ml) (Optical Density)	**Salt concentration (**1.0 mg/ml) (Optical Density)
Streptococcus sp. C1	0.590	0.300	0.200	0.113	0.060
Streptococcus sp. C2	0.380	0.230	0.189	0.090	0.010

Indication: Strong producer more than 0.240 nm; weak producer between 0.120-0.240 nm while non-producer less than 0.120 nm.

Table 4: Effect of salt concentration on biofilm formation

Isolate	pH 6.4 (Optical Density)	pH 7.4 (Optical Density)	pH 8.4 (Optical Density)
Streptococcus sp. C1	0.600	0.580	0.210
Streptococcus sp. C2	0.570	0.530	0.190

Indication: Strong producer more than 0.240 nm; weak producer between 0.120-0.240 nm while non-producer less than 0.120 nm.

Antibacterial activity of Walnut stem bark, Cinnamon and Clove extracts

Antibacterial activity of benzene, methanol and aqueous extracts of stem bark of walnut, cinnamon and clove buds were studied against the *Streptococcus* sp. C1 and C2 isolated from oral samples of diabetes type 2 patients by disc diffusion method. The results obtained clearly indicated that the aqueous extract of clove displayed the highest antibacterial activity against *Streptococcus* sp. C1 i.e. 28 mm zone of inhibition followed by benzene and methanol. The aqueous extract of walnut stem bark showed the lowest antibacterial activity against *Streptococcus* sp. C1 and C2 i.e. 7 mm zone of inhibition each (Table 5). Antimicrobial activities of various other parts of *Juglans regia* (walnut) have already been studied with different microorganisms. Fadi Qa'dan *et al*. (2005) studied the antimicrobial activity of *Juglans regia* leaf extracts, in which they have reported the zone of inhibition ranged from 15.8–17.6 mm against *P. acnes*, 11.3–15.7 mm against *S. aureus* and 12.9–15.5 mm against *S. epidermidis* by disc diffusion method. Coban and Bivik (2010) studied the antimicrobial activity of ethanol extracts of *Juglans regia* (walnut) leaves against 12 bacteria and the results showed that the ethanol extracts of *Juglans regia* inhibited the growth of nine bacteria and the inhibition zones ranged between 12–25 mm. Upadhyay *et al*. (2010) reported the in vitro antifungal activity of petroleum ether, benzene, chloroform, acetone and methanol extracts of stem bark of *Juglans regia*. Noumi *et al*. (2011) reported that *J. regia* leaves extracts were most effective against Gram positive and Gram negative bacteria than the extracts of *S. persica*. Deshpande *et al*. (2011) reported the antimicrobial assay of the aqueous and acetone extracts of *J. regia* L. on the salivary microflora and showed that acetone extract had significant inhibitory effect on the growth of microorganisms.

Table 5: Antimicrobial assessment benzene, methanol and aqueous extracts of walnut stem bark, cinnamon and clove buds on *Streptococcus* sp. C1 and C2.

Plant Extracts	*Streptococcus* C1			*Streptococcus* C2		
	Zone of inhibition (mm)	MIC mg/mL	MBC mg/mL	Zone of inhibition (mm)	MIC mg/mL	MBC mg/mL
Benzene- walnut stem bark	10	1.260	0.988	11	1.077	0.930
Benzene- cinnamon	12	1.010	0.868	19	0.186	0.081
Benzene- clove	22	0.090	0.079	12	1.020	0.858
Methanol-walnut stem bark	12	1.010	0.868	15	0.920	0.747
Methanol-cinnamon	11	1.087	0.940	13	1.080	0.898
Methanol-clove	20	0.146	0.070	23	0.070	0.059
Aqueous-walnut stem bark	7	2.013	1.041	7	2.012	1.051
Aqueous-cinnamon	14	0.987	0.762	14	0.987	0.762
Aqueous- clove	28	0.067	0.047	22	0.090	0.079
Antibiotics-Tetracycline	29	-	-	30	-	-

Saraf *et al*. (2011) reported that maximum zone of inhibition was formed by butanolic seeds extract of *C. zeylanicum* (cinnamon species) against *Staphylococcus aureus* followed by *Streptococcus species*, while very low inhibition was shown by *Shigella sonii* and *K. pneumoniae* at a concentration of 100µg/ml. It was reported that euginol and cinnamic aldehyde extracted from clove and cinnamon, exhibit high activity against broad spectrum of bacteria (Moleyer and Narasimhan, 1992; Cowan, 1999; Alexander and Richard, 2004; Abu-Shanab *et al*., 2004; Yeh *et al*., 2009). The primary function of clove oil in the clove paste in aphthous ulcer treatment act as a biocide, being active against invasive bacteria, fungi and even invading larvae (Holloway *et al*., 2004).

Minimum Inhibitory Concentration

The MIC and MBC values of benzene, methanol and aqueous extracts of stem bark of walnut, cinnamon and clove buds were studied against the *Streptococcus* sp. C1 and C2 isolated from oral samples of diabetes type 2 patients were showed in table 5. The best MIC and MBC against *Streptococcus* C1 were 0.067 and 0.047 for aqueous extract of clove respectively, and against *Streptococcus* C1 were 0.070 and 0.059 for methanolic extract of clove, respectively.

CONCLUSION

From present study it can be concluded that *Streptococcus* sp. isolated from oral samples of diabetic type 2 patients form biofilm for their defense. The results of the antibacterial activity indicate that the aqueous extract of clove exhibits highest antibacterial activity against the isolated biofilm forming bacteria. Thus the tooth pastes which contains clove extract to diabetic type 2 patients should be recommended.

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No potential conflict of interest was reported by the author.

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