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Research Article

HPTLC ANALYSIS OF *ECLIPTA PROSTRATA* AND *PSIDIUM GUAJAVA* EXTRACTS AND THEIR EFFECT ON CELL-SURFACE HYDROPHOBICITY OF A CONSORTIUM OF DENTAL PLAQUE ISOLATES

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ABSTRACT

Objective: The present study elucidates the preliminary phytochemical analysis and High Performance Thin Layer Chromatography (HPTLC) fingerprint analysis of Hot Aqueous-Ethanolic Extract (HAEE) of the leaves of *Psidium guajava* and Cold Aqueous-Ethanolic Extract (CAEE) of the leaves of *Eclipta prostrata* and evaluates their effect on cell-surface hydrophobicity of a consortium of dental plaque isolates.

Methods: The extracts were evaluated for their effect on cell-surface hydrophobicity by the Microbial Adhesion to Hydrocarbon (MATH) assay. The extracts were further subjected to preliminary phytochemical screening by standard procedures and High Performance Thin Layer Chromatography (HPTLC) analysis.

Results: The test consortium was found to have an inherent hydrophobicity index (HPBI) > 70% thus classifying it as hydrophobic. The hydrophobicity of the test consortium significantly reduced in the presence of various concentrations of both extracts evaluated, in a concentration dependent manner. In the presence of *P.guajava* HAEE, the HPBI was significantly reduced at concentrations of 1 and 2.5 mg ml $^{-1}$, while *E. prostrata* CAEE significantly reduced HPBI at concentrations between 1.25 to 7.5 mg ml $^{-1}$. Phytochemical screening and HPTLC analysis of the extracts revealed a strong presence of flavonoids and tannins among other phytoconstituents.

Conclusion: Flavonoids and tannins detected in *P.guajava* HAEE and *E. prostrata* CAEE may be responsible for the observed effect on cell surface hydrophobicity of the test consortium. Such extracts capable of modulating cell surface hydrophobicity can be of great significance in oral care research.

Keywords: Cell surface hydrophobicity, Dental plaque isolates, HPTLC finger printing

INTRODUCTION

Dental biofilms or 'plaque' is a film of micro-organisms like *Streptococcus mutans*, *Streptococcus sanguis, Streptococcus mitis*, etc found on the tooth enamel, embedded in a matrix of polymers of salivary and bacterial origin. [1] When allowed to proliferate and mature unrestricted, these biofilms often lead to deterioration of oral health [2] and indirectly affect systemic health. [3]

The onset of dental biofilm formation is characterised by the initial adhesion of oral bacteria to the tooth surface and their subsequent colonization. Teeth are covered by an acquired pellicle, formed by selective adsorption of salivary components onto the enamel. [4] The initial attachment of bacteria to teeth is therefore thought to involve specific (adhesin-receptor) and non specific interactions between surface components of the organism and immobilized salivary components of the acquired pellicle. [4] Among non specific interactions, hydrophobic interactions are important for attachment of bacteria to the surfaces as well as to each other. [2,4-7] The cell-surface hydrophobicity (CSH) is dependent on multiple factors like presence of cell wall appendages, presence of lipoglycan lipoteichoic acid and structurally related polypeptides within the cell wall. [8-10]

Since CSH is repeatedly implicated as being crucial during early adhesion, it is now being explored as a target in anti-caries research. Many natural products have been evaluated for their potential to reduce CSH and in turn impede adherence of cariogenic bacteria. [2, 6, 10-12]

In the present study, two medicinal plants - *Eclipta prostrata* Linn and *Psidium guajava* Linn were investigated for their ability to reduce CSH of a test consortium of dental plaque isolates. *E.prostrata* (Family: Asteraceae) is a small evergreen tree possessing anti-inflammatory, antimicrobial, antiviral, analgesic and immunomodulatory properties. [13] *P.guajava* (Family: Myrtaceae), is a small tree, various parts of which exhibit antioxidant, hepatoprotective, antimicrobial and antidiabetic properties. [14] Studies have reported the effect of aqueous extract of *P.guajava* on reducing CSH of different oral bacteria [12] as well as the effect of Guaijaverin, a flavonoid isolated from methanolic extract of *P. guajava,* on CSH of *Streptococcus mutans*. [11] *P.guajava* and *E. prostrata* have both been previously studied for anti-plaque activity in which, Hot Aqueous-Ethanolic Extract (HAEE) of the leaves of *Psidium guajava* and Cold Aqueous-Ethanolic Extract (CAEE) of the leaves of *Eclipta prostrata* were found to be the most effective [15] and hence selected for evaluation for their effect on CSH in this study.

The aim of the present study was to evaluate the selected plant extracts for their ability to influence CSH of the test consortium and to analyse the extracts for the presence of phytochemicals by preliminary phytochemical screening and HPTLC.

MATERIALS AND METHODS

Brain Heart Infusion medium (BHI) was purchased from Himedia Laboratories, Mumbai, India. Chemicals used were of analytical grade purchased from Qualigens Fine Chemicals, Mumbai, India. The TLC silica plates were purchased from Merck, Darmstadt, Germany.

Preparation of plant extracts

Young leaves of *E. prostrata* and *P. guajava* were collected from Mumbai, India. The plants were authenticated by The Blatter Herbarium, Mumbai, India. The leaves were washed, dried and pulverized into a powder. For preparation of CAEE of *E.prostrata*, powdered plant material was macerated in Aqueous-Ethanol (1:1) with intermittent shaking for 2 days. For preparation of HAEE of *P.guajava*, powdered plant material was subjected to soxhlation for 6 hours in aqueous-ethanol (1:1). The extracts were filtered, dried at 50^{o}C and stored at 4^{o}C until further use.

Microorganisms

The test consortium was prepared as previously described. [15] *Streptococcus mutans* MTCC#890 and *Streptococcus mitis* MTCC#2696 were obtained from Institute of Microbial Technology, Chandigarh, India. 26 different isolates were obtained from plaque

samples of 10 healthy volunteers, of which 6 isolates capable of forming biofilms were used as a part of the test consortium along with standard strains - *S.mitis* and *S.mutans*. As per Bergey's manual of Systematic Bacteriology [16], isolates were identified using conventional biochemical tests up to species level as *Streptococcus salivarius, Streptococcus mitior, Streptococcus sanguinis* and *Streptococcus milleri* while two isolates were identified up to genus level as *Streptococcus* spp. Working cultures were prepared by inoculating all strains of test consortium onto fresh Brain Heart Infusion agar and incubating overnight at 37°C in candle jar.

Effect of extracts on cell-surface hydrophobicity of test consortium

CSH of test consortium was measured according to Microbial Adhesion Test to Hydrocarbon (MATH) assay as described by Martin et al. [17] with slight modifications. CSH was determined using toluene as a hydrocarbon to mimic the hydrophobic nature of the acquired pellicle. [11,12]

Cells grown in BHI medium with various concentrations of extract (ranging from 0.25 to 2.5 mg ml $^{-1}$ for *P.guajava* HAEE and from 1.25 to 7.5 mg ml $^{-1}$ for *E. prostrata* CAEE) were washed twice, suspended in sterile saline (0.85%). 3ml of cell suspension was placed in tubes and 0.25 ml of toluene was added. The tubes were vortex mixed for 2 min and allowed to equilibrate at room temperature for 10 min. After toluene phase was separated from aqueous phase, OD of the aqueous phase was determined spectrophotometrically at 600 nm.

The following controls were set up: Test consortium + BHI medium, denoted by (C); Test consortium + BHI medium + Solvent (Aqueous-Ethanol) denoted by (C+AE). The controls were set up and processed as described previously but without the addition of extracts to allow determination of relative CSH of the test consortium in the absence of extracts. The hydrophobic index was calculated as: (OD initial – OD final)/OD initial*100%. Hydrophobic index greater than 70% was arbitrarily classified as hydrophobic. [17]

Statistical analysis

Statistical analysis was performed using GraphPad Prism®5 software. Experiments were carried out in triplicate. The data was analyzed using ANOVA followed by Dunnett's multiple comparison test. P value ≤ 0.05 was considered significant.

Phytochemical Analysis

Phytochemical screening of the extracts was carried out to detect the presence of Flavonoids, Alkaloids, Carbohydrates, Glycosides, Steroids, Tannins and Proteins using standard chemical tests. [18, 19]

HPTLC analysis

Preparation of sample solutions

P.guajava HAEE and *E. prostrata* CAEE were accurately weighed and dissolved in Aqueous- Ethanol (1:1) such as to obtain sample solutions of concentration 30 mg ml $^{-1}$ and 50 mg ml $^{-1}$ respectively.

HPTLC was performed on 10 × 10 cm plates coated with 0.25 mm layer of silica gel 60 F $_{254}$ (Merck, Germany). The sample solutions were applied with bandwidth of 8 mm using a CAMAG (Muttenz, Switzerland) Linomat V sample applicator equipped with 100 µl Hamilton Syringe. A constant application rate of 100 nl sec $^{-1}$ was maintained. The plate was air dried and kept for development up to 80 mm in pre-saturated CAMAG twin trough developing chamber containing 10 ml of solvent system- Ethyl acetate: Ethanol: Water (6: 4: 4). After drying, the spots were visualized under CAMAG UV cabinet (254 and 366 nm). Then the plate was scanned using CAMAG TLC scanner equipped with WINCATS software (CAMAG). The presence of flavonoid and tannin constituents was confirmed by chemical derivatization, where the developed plate was immersed in a developing chamber with 1% ethanolic solution of $AlCl_3$ and 10% aqueous $FeCl_3$ respectively. [19]

RESULTS

Effect of extracts on cell-surface hydrophobicity of test consortium

Presence of the extracts of *P.guajava* and *E. prostrata* influenced cell surface hydrophobicity of the test consortium in a dose dependent manner. The inhibitory effect of various concentrations of *P.guajava* HAEE and *E. prostrata* CAEE on hydrophobicity index (HPBI) have been summarised in Fig. 1.

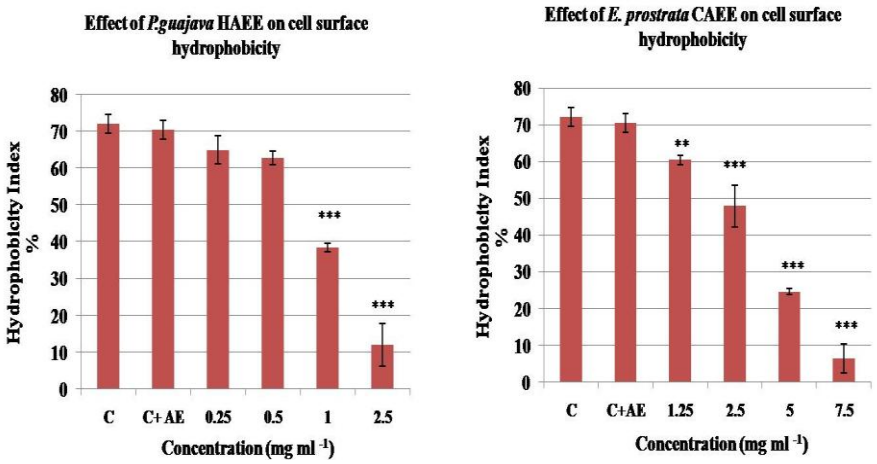

Fig. 1: It shows the effect of various concentrations of *P.guajava* HAEE and *E. prostrata* CAEE on cell surface hydrophobicity of test consortium. (C) and (C+AE) denote controls. The percentages were mean ± S.D. of three determinations. (n=3; *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001)

HPBI of the test consortium was found to be 72.1%, which is indicative of its hydrophobic nature. In the presence of *P.guajava* HAEE, the HPBI was significantly reduced at concentrations of 1 and 2.5 mg ml $^{-1}$, while *E. prostrata* CAEE significantly reduced HPBI at concentrations between 1.25 to 7.5 mg ml $^{-1}$, when compared with solvent control (C+AE)

Phytochemical screening

The preliminary phytochemical screening of *P.guajava* HAEE and *E. prostrata* CAEE by chemical tests is summarised in Table 1. The extracts exhibited a strong presence of flavonoids and tannins among other phytoconstituents.

High Performance Thin Layer Chromatography (HPTLC) analysis

A densitometric HPTLC analysis was performed to obtain a characteristic finger print profile of *P.guajava* HAEE and *E. prostrata* CAEE at 254 nm and 366 nm as depicted in Fig. 2-5. The Rf values and peak areas are recorded in Tables 2 and 3.

Table 1: It shows the phytochemical constituents detected in preliminary phytochemical screening of *P.guajava* HAEE and *E. prostrata* CAEE

	Phytochemical constituents	*Psidium guajava* HAEE	*Eclipta prostrate* CAEE
1	Carbohydrates	+	+
2	Proteins	-	+
3	Steroids	+	+
4	Cardiac glycosides	+	+
5	Anthraquinone glycosides	+	-
6	Flavonoids	++	++
7	Alkaloids	+	+
8	Tannins	++	++

++ = detected in appreciable quantity; + = detected in low quantity; - = not detected

Table 2: It shows the peaks obtained for HPTLC analysis of *P.guajava* HAEE with Rf values and % Area

254 nm			**366 nm**		
Peak	**Rf**	**%Area**	**Peak**	**Rf**	**%Area**
1	0.01	1.86	**1**	0.23	0.36
2	0.10	0.46	**2**	0.48	0.68
3	0.20	8.46	**3**	0.55	2.84
4	0.48	31.08	**4**	0.64	9.99
5	0.61	25.98	**5**	0.69	9.44
6	0.71	23.06	**6**	0.74	20.28
7	0.87	9.10	**7**	0.86	56.42

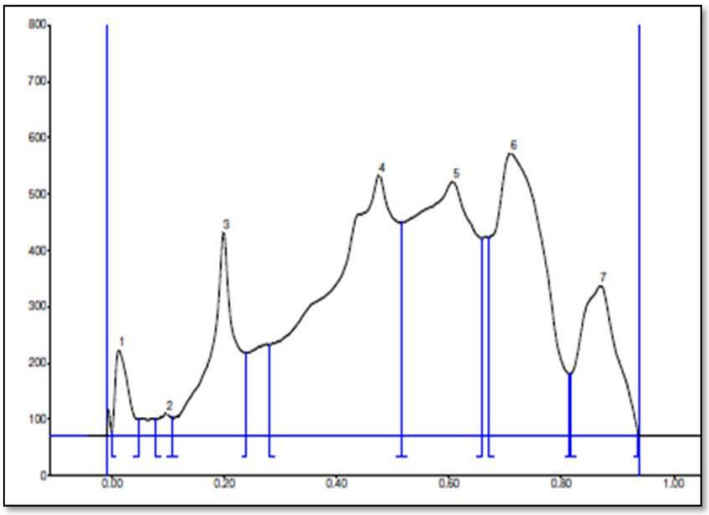

Fig. 2: It shows the HPTLC peak densitogram display of *P.guajava* HAEE at 254 nm

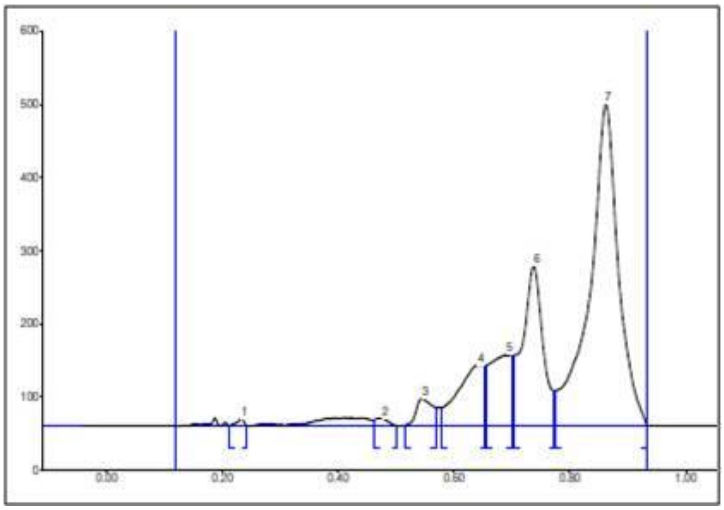

Fig. 3: It shows the HPTLC peak densitogram display of *P.guajava* HAEE at 366 nm

Table 3: It shows the peaks obtained for HPTLC analysis of *E. prostrata* CAEE with Rf values and % Area

254nm			366 nm		
Peak	**Rf**	**%Area**	**Peak**	**Rf**	**%Area**
1	0.12	4.91	**1**	0.13	0.71
2	0.15	5.41	**2**	0.17	0.70
3	0.30	6.97	**3**	0.23	3.52
4	0.46	12.00	**4**	0.32	4.60
5	0.52	7.85	**5**	0.50	4.83
6	0.60	12.46	**6**	0.56	6.68
7	0.63	5.10	**7**	0.64	8.00
8	0.67	5.63	**8**	0.69	9.61
9	0.76	11.07	**9**	0.74	4.63
10	0.86	28.59	**10**	0.81	15.57
			11	0.87	27.43
			12	0.93	13.71

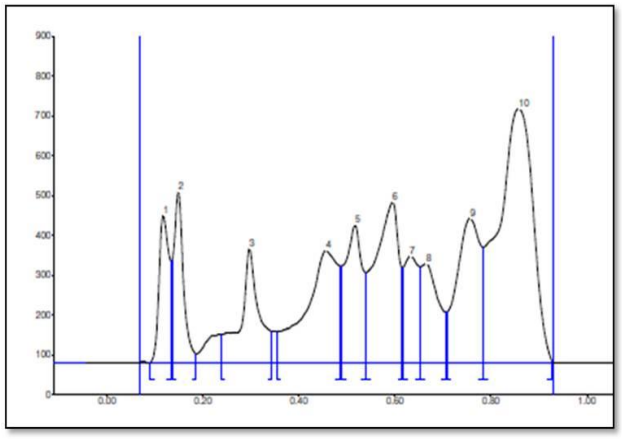

Fig. 4: It shows the HPTLC peak densitogram display of *E. prostrata* CAEE at 254 nm

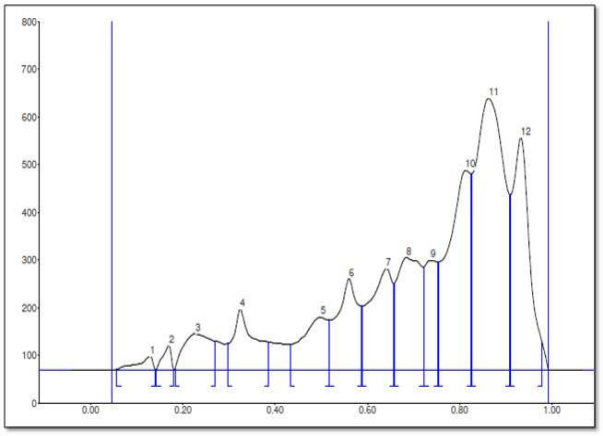

Fig. 5: It shows the HPTLC peak densitogram display of *E. prostrata* CAEE at 366 nm

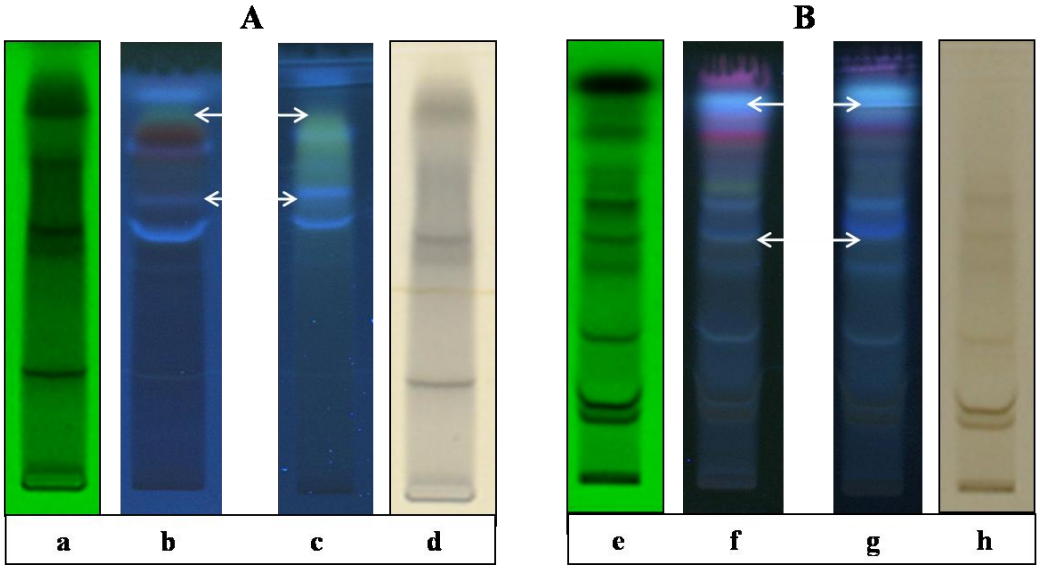

Fig. 6: It shows chromatograms of (A) *P. guajava* HAEE, (a) Under UV 254 nm (b) Under 366 nm, (c) After derivatization with 1% alcoholic $AlCl_3$ under 366 nm (d) After derivatization with 10% aqueous $FeCl_3$ under day light and chromatograms of (B) *E. prostrata* CAEE, (e) Under UV 254 nm (f) Under 366 nm, (g) After derivatization with 1% alcoholic $AlCl_3$ under 366 nm (h) After derivatization with 10% aqueous $FeCl_3$ under day light. Arrows indicate the intensification of fluorescence post derivatization 1% alcoholic $AlCl_3$ under 366 nm

As seen in Figure 6(A) (c), post derivatization with 1% alcoholic $AlCl_3$, the blue fluorescence of band 4 (Rf = 0.64) and yellow fluorescence of band 6 (Rf = 0.74) intensified, while Figure 6(B) (g), showed a similar result for blue fluorescence of bands 5 (Rf = 0.5) and 11 (Rf = 0.87). This intensification of fluorescence is characteristic of flavonoids. Figure 6(A) (d) and 6(B) (h) showed appearance of distinct brown-black bands after derivatization with 10% aqueous $FeCl_3$ which demonstrated the presence of tannins.

DISCUSSION

For any organism to colonise the oral cavity, its ability to adhere is indispensible in order to withstand mechanical forces exerted by cheek and tongue muscles as well as the salivary flow in the mouth [2]. Hydrophobic bond interactions have been implicated as a contributing factor to facilitate this adherence to the tooth enamel surface [4, 8, 9, 20] which may subsequently lead to build up of plaque in the absence of oral hygiene. Anti-plaque agents, which inhibit hydrophobic bond formation, would interfere with the adherence of cariogenic bacteria thereby promoting oral health. [12] Since reports of side effects of antibacterial agents like chlorhexidine and cetylpyridinium chloride have surfaced, there has been a renewed interest in phytotherapy as an alternative. [21]

In the present study, quantitative determination of CSH using MATH assay provided information about the inherent CSH of the test consortium and the resultant change in hydrophobicity after exposure to the plant extracts. Based on the HPBI value of 72.1%, the test consortium can be classified as hydrophobic. [17] In the presence of various concentrations of *P.guajava* HAEE and *E. prostrata* CAEE, CSH of the test consortium was markedly reduced in a concentration dependent manner. *P.guajava* HAEE and *E. prostrata* CAEE have minimum inhibitory concentration (MIC) of 1 mg ml^{-1} and 5 mg ml^{-1} respectively against the test consortium. [15] This suggested that *E. prostrata* CAEE showed significant reduction in CSH even at sub-MIC levels. Results of crude extracts of *Helichrysum italicum* and *Psidium guajava* interfering with CSH of oral bacteria have been reported by Nostro et al. [12] and Fathilah et al. [10] respectively. Prabhu et al. have demonstrated similar results with Guaijaverin, a flavonoid isolated from *Psidium guajava* [11] while Okada et al. elucidated the ability of polyphenols from Cranberry to reduce CSH. [6]

Since the potential of *P.guajava* HAEE and *E. prostrata* CAEE to influence CSH may be attributed to the biologically active components they contain; it is essential that these extracts be screened to detect the presence of different phytochemicals that may in turn help to explain their mode of action. HPTLC has proven to be an important tool for the study and evaluation of such botanical materials of therapeutic value. Phytochemical screening and HPTLC analysis of *P.guajava* HAEE and *E. prostrata* CAEE revealed the strong presence of polyphenols like flavonoids and tannins, consistent with prior studies. [13, 14, 22-25] These polyphenols have been reported to affect CSH. [6, 11, 26] CSH is associated with cell-surface proteins [8] and hence it is possible that the active components of *P.guajava* HAEE and *E. prostrata* CAEE bind or mask cell-surface proteins resulting in a reduction of CSH. [6, 11, 12]

Swanberg et al have reported that more hydrophobic strains of cariogenic bacterium *Streptococcus mutans* implanted much better in the oral cavity as compared to less hydrophobic strains [27] while Nesbitt et al have demonstrated that the presence of hydrophobic bond inhibitors significantly reduced adherence of *Streptococcus sanguis* to saliva coated hydroxylapatite. [28] Hence there is evidence to suggest that the observed reduction of overall CSH of test consortium by the extracts would definitely contribute to impeding adherence of oral bacteria to teeth and reducing incidence of dental plaque and caries.

Majority of oral care research is directed towards development of natural and synthetic bactericidal agents; however the mode of action of these agents may result in development of selective pressure and overgrowth of resistant bacteria. [2] Interfering with bacterial adherence by modulating CSH can be a promising alternative to prevent plaque build up without disrupting the homeostasis in the oral bio-system.

CONCLUSION

HAEE of *Psidium guajava* and CAEE of *Eclipta prostrata* proved to be effective in reducing CSH of dental plaque bacteria which would in turn affect their ability to adhere to tooth enamel. The data gathered in this study may be used to develop nature based oral care products that are as effective as their synthetic counterparts and additionally aid in circumventing the side effects of the same.

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