ORIGINAL RESEARCH

Evaluation of the effects of Albizia lebbeck bark extracts on the cariogenic properties of Streptococcus mutans

Shashidara Raju, Archana Vaikom Krishnan, Mukund Raghunath Nair, Austin Richard

Abstract

Introduction: Oral diseases continue to be a major and common health problem worldwide and affect the quality of life by causing considerable pain and discomfort. While the mechanistic methods of caries prevention and treatment are in vogue, the concern about adverse effects of the constituents of these methods persists. Thus, there has always existed a need to develop effective and safe strategies to combat oral pathogens such as Streptococcus mutans (S. mutans).

Aim: To examine the action of Albizia lebbeck (A. lebbeck) bark extracts on the growth and virulence factors of S. mutans such as- biofilm formation, surface adherence, cell surface hydrophobicity and acid production.

Materials and Methods: Ethyl acetate, hexane, and chloroform extracts of the bark of A. lebbeck was prepared and tested for their activity against the ATCC 25175 S. mutans. The phytochemical constituents of the extracts were determined by biochemical assays and the MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) were obtained using the well diffusion assay. The effect of the sub-MIC concentrations of extracts on biofilm formation & eradication, microbial growth, adherence of the strain to glass surfaces, cell surface hydrophobicity and acid production were also determined.

Results: All extracts inhibited the organism's growth, and MIC was determined as 25 mg/ml. the sub-MIC concentrations of the extracts were found to- inhibit the formation of biofilms, eradicate formed biofilms and interfere with the adherence and acid production of S. mutans.

Conclusion: The ethyl acetate and ethanolic extracts were found to be active at low concentrations (0.02mg/ml) and demonstrate a potential to be used in the formulation of anti-caries preparations.

Keywords: Dental Caries, Bio-films, MIC/MBC, Ethnomedicine

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Introduction

Oral diseases continue to be a major and common health problem worldwide, affecting the quality of life by causing considerable pain and discomfort.[1] Dental caries and periodontal diseases are amongst the two most common oral diseases prevalent across the Globe. In India, the dental caries incidence ranges from 49%-89% across different age groups while the global caries burden is estimated to only be around 36%. [2,3]

The normal oral flora consists of between 700-1000 species of microorganisms and the occurrence of many oral diseases is thought to be a result of oral dysbiosis.[2] Dental caries is one such disease with the etiological factors leading to a dysbiosis in the dental plaque, favouring the growth and development of cariogenic organisms. *S. mutans* is amongst the most common bacteria found in cariogenic biofilms and plays a major role in the initiation and progression of dental caries on tooth surfaces by various modes. *S. mutans* is amongst the earliest colonizers of dental plaque and creates a microenvironment detrimental to the growth of other micro-organisms by the formation of adherent biofilms and acid production that inhibits the growth of other species by lowering the plaque pH.

The easy accessibility of the oral cavity to mechanical cleansing methods has meant that these methods have

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been given a lot of impetus in caries control. However, the inability of mechanical methods to cleanse all tooth surfaces, the possibility of micro-abrasion and emerging evidence for successful chemical plaque control, have led to an increased interest in the latter.[3]

Chlorhexidine gluconate and fluorides in various forms are the mainstays of chemical plaque control currently, they however face challenges of non-selective action, drug resistance, adverse effects, and possible toxicity.[4]

The field of phytochemicals and ethnomedicine focuses on the use of natural pharmaceutical agents and also the ethnic groups from which these agents are derived from and has given numerous naturally occurring compounds with medicinal uses. *A.lebbeck* is a plant used by the Malayali tribals of the Kolli hills, as a therapeutic twig for oral health.[5-7] Extracts of this plant have been proven to have no cytotoxic effects against normal human cell lines.[8,9] This study investigates the potential anti-microbial uses of extracts of the plant *A.lebbeck against S. mutans,* and the effects of the extracts against the other virulence factors of *S. mutans* i.e. biofilm formation, acid production, cell surface hydrophobicity, adherence and growth in presence of sucrose.

Materials and Methods

a. Sample collection - Collection of plant material

The barks of *A. lebbeck* were collected from various places in and around the Manasagangothri region of Mysuru & Chamarajanagar districts. They were identified and authenticated by a botanist. The barks were rinsed first under running tap water, then in sterile distilled water and shade dried over a period of 3-4 weeks. A coarse powder of the dried bark was obtained by hand crushing individual pieces separately and the fine powder subsequently prepared using a mixer grinder was stored in airtight plastic bottles at 4°C.

b. Extraction and Preparation of the Herbal Extract

The bark powder was extensively extracted using the following solvents of increasing polarity (60-80^{0}C) using a Soxhlet apparatus- Hexane, Chloroform, Ethyl acetate and Ethanol. The extracts were then filtered using Whatman no.2 filter paper. The filtrate thus obtained was concentrated using

Fig. 1: MSB agar plates with ethyl acetate extract showing inhibition zone, none of the other extracts showed any inhibition zones

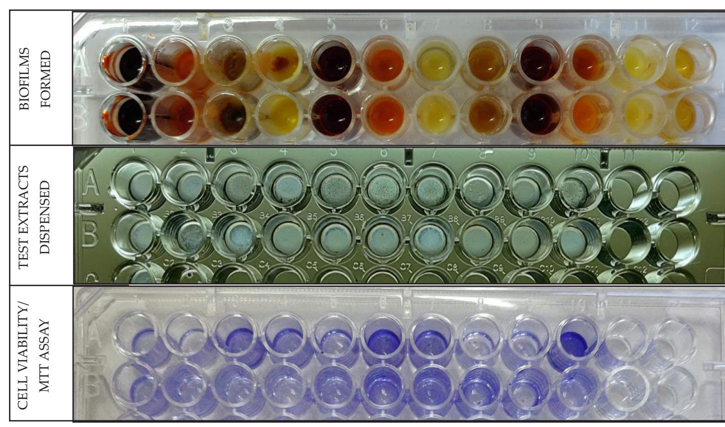


Fig. 2: Inhibition of biofilm formation of S. mutans by various extracts and MTT assay for biofilm eradication

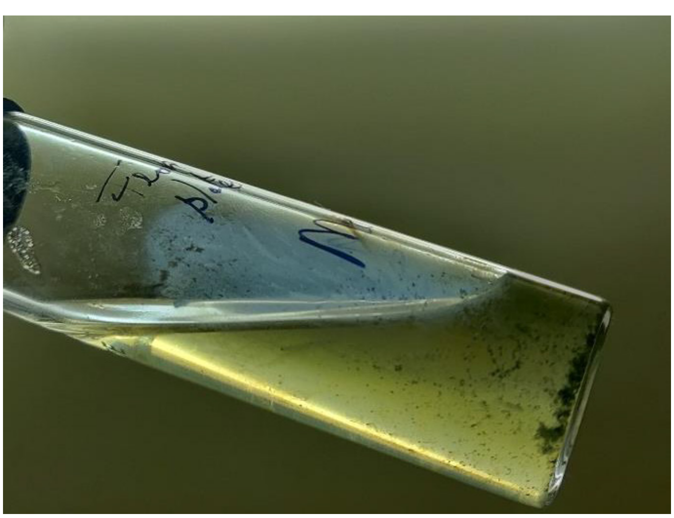


Fig. 3: Test for adherence to the glass surface

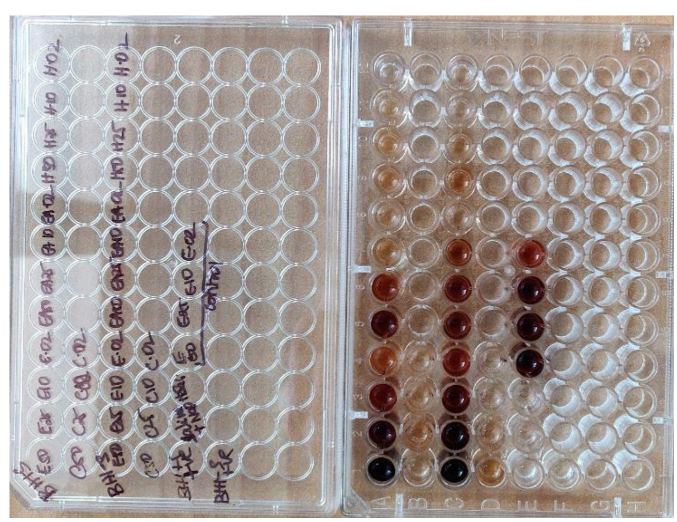


Fig. 4: Microplating for measuring the effect on growth

a vacuum evaporator. All Solvents used were of Laboratory Reagent (LR) grade.

c. Phytochemical screening and identification of constituents

The extracts were tested for the presence of alkaloids, phenolic compounds, flavonoids, and carbohydrates using Sonnenschein reagent, Ferric Chloride, Sodium Hydroxide and Benedict's reagent respectively. The presence of proteins was analysed by the biuret test and the Salkowski test was used to detect the presence of terpenoids. The total antioxidant activity was also determined by the Chronoamperometric method using Ascorbic acid as a standard

Table 1: Phytochemical analysis of *A.lebbeck* extract

Extract / Phyto chemical	Ethanol extract	Ethyl acetate	Chloro-form	Hexane
Alkaloids	-	-	+	-
Phenolics	+	-	-	-
Flavonoids	-	+	+	+
Carbohydrates	+	+	+	+
Proteins	+	-	-	-
Terpenoids	-	-	+	-

Table 2: Mean zones of inhibition of various extracts of *A.lebbeck* against *S.mutans* at various concentrations

Concentra-tion (mg/ml)	Hex-ane	Ethyl acetate	Ethanol	Chloro-form
100	0	31.6667 ±1.820	0	0
75	0	29.6667 ±1.729	0	0
50	0	26.6667 ±1.729	0	0
25	0	12.5±1.684	0	0

Table 3: Mann Whitney U test comparing the effects of various extracts on biofilm formation of S.mutans at various concentrations in the absence of sucrose

Biofilm without sucrose	Mean	SD	Median	Z	Significance
Ethanol 10mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethanol 5mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethanol 2mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethanol 0.02mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 10mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 5mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 2mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 0.02mg	.04967	.009452	0.073	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Hexane 10mg	.05933	.006807	0.057	-1.964	0.046(S)
Control	.37367	.025794	0.381		
Hexane 5mg	.06367	.002309	0.065	-1.964	0.046(S)
Control	.37367	.025794	0.381		
Hexane 2mg	.06533	.000577	0.065	-1.993	0.050(N.S)
Control	.37367	.025794	0.381		
Hexane 0.02mg	.25967	.080928	0.217	-1.993	0.050(N.S)
Control	.37367	.025794	0.381		
Chloroform 10mg	.07933	.003215	0.078	-1.528	0.127(N.S)
Control	.37367	.025794	0.381		
Chloroform 5mg	.27533	.005774	0.117	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		
Chloroform 2mg	.12533	.018930	0.272	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		
Chloroform 0.02mg	.25679	.003215	0.381	-1.993	0.046(S)
Control	.37367	.025794	0.381		

d. Assessing the antimicrobial activity against Streptococcusmutans (MIC)

S.mutans strain (ATCC25175) was obtained from Chromachemie Laboratory Private Limited, Bengaluru. The reconstitution procedure was done,as per instructions of the provider, and the growth was verified by culture and gram staining characteristics. Multiple aliquots were prepared and kept for further use. The MIC of the test compound was determined by the NCCLS guidelines (National Committee for Clinical Laboratory Standards 2000).[10] A 24 hour inoculum of the organism cultured on BHI (Brain Heart Infusion Media) and MSA (Mitis Salivarius Agar), was adjusted in sterile saline to the McFarland 0.5 standard to yield a cell density of 1.5x 108 CFUs/ml and plated using lawn culture technique on BHI agar. On the agar, wells were cut using a heat sterilized 5mm well borer and 50µl of each test solution at concentrations of 100mg/ml (100%), 75 mg/ml (75%), 50mg/ml (50%) and 25mg/ml (25%) was dispensed into the wells. Positive growth controls, sterility controls and negative controls (steriledistilled water, DMSO and all solvents) were also incubated along with the test plates at 37±2^{0}C for 24 hours. Following incubation, the antimicrobial activity was read as a measure of zones of inhibition around each well.

e. Determination of Biofilm Inhibition:

250 µl aliquots of overnight cultures of S. mutans were added to pure BHI broth and adjusted to the 0.5 McFarland

Table 4: Mann Whitney U test comparing the effects of various extracts on biofilm formation of S.mutans at various concentrations in the presence of sucrose

Biofilm with sucrose	Mean	SD	Median	Z	Significance
Ethanol 10mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethanol 5mg	.00000	.009539	0.352	-1.091	0.037(S)
Control	.37367	.025794	0.381		
Ethanol 2mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethanol 0.02mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 10mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 5mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 2mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Ethyl acetate 0.02mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.37367	.025794	0.381		
Hexane 10mg	.11167	.005774	0.115	-1.993	0.046(S)
Control	.37367	.025794	0.381		
Hexane 5mg	.21933	.006807	0.217	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		
Hexane 2mg	.24433	.011547	0.251	-1.993	0.046(S)
Control	.37367	.025794	0.381		
Hexane 0.02mg	.23500	.010000	0.235	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		
Chloroform 10mg	.21133	.004933	0.209	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		
Chloroform 5mg	.20267	.021455	0.213	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		
Chloroform 2mg	.22900	.015875	0.223	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		
Chloroform 0.02mg	.27200	.010000	0.272	-1.964	0.050(N.S)
Control	.37367	.025794	0.381		

standard to yield a cell density of 1.5x 108 CFUs/ml. To this 3% supplemental horse serum was added. The adjusted inoculum and sub-MIC concentrations of the extracts were dispensed in wells of an untreated 96-welled polystyrene microtiter plate as triplicates in the presence and absence of 5% sucrose and incubated at 37±2^{0}C for 24 hours. The wells were then gently aspirated to remove their contents, washed with Phosphate Buffered Saline (PBS at pH 7.4) three times to remove planktonic cells and left to air dry at room temperature. The biofilms were then fixed with 99% methanol for 15 min, air-dried, stained with 1% crystal violet for 20 min, rinsed carefully under running water and dried. The fixed dye was extracted with 30% glacial acetic acid and dispensed into sterile wells of a new microtiter plate to obtain optical density readings and quantify the biofilms.

f. Determination of Biofilm Eradication (Minimum biofilm eradication concentration- MBEC)

The capability of the extracts to eradicate already formed biofilms was tested using the MTT[(3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide)]cell viability assay. Biofilms of S.mutans were prepared by incubating 100µl of McFarland 0.5 (1.5x 108 CFUs/ml) adjusted strains in the wells of a microplate according to Dias-Souza MVet al for 24 hours at 37±2°C(11). The supernatant was disposed and to the formed biofilms, 100µl aliquots of each dilution of extract was dispensed and incubated at 37±2°C for 24 hours with positive and negative controls. Each concentration of extract along with the plain broth was also dispensed as controls to allow for elimination of bias caused due to the color of the extract

Table 5: Unpaired t-test for comparison of biofilm eradication of A. lebbeck extracts

Extract concen-tration		Mean	t-value	p-value
10 mg	Ethanol	0.061±.0032	4.4250	< 0.001 HS
	Ethyl acetate	0.043 ±.0061		
	Hexane	0.002±.0032		
	Chloro-form	0.011±.0045		
	Positive Control	0.406±.04574		
5 mg	Ethanol	0.002±.0006	416.7330	< 0.001 HS
	Ethyl acetate	0.004±.0002		
	Hexane	0.002±.0		
	Chloro-form	0.003±.0		
	Positive Control	0.406±.04574		
2 mg	Ethanol	0.021±.0032	41.5263	< 0.001 HS
	Ethyl acetate	0.003±.0		
	Hexane	0.002±.0		
	Chloro-form	0.001±.0		
	Positive Control	0.406±.04574		
.02 mg	Ethanol	0.025±.002	34.1304	< 0.001 HS
	Ethyl acetate	0.005±.0		
	Hexane	0.002±.0		
	Chloro-form	0.003±.0		
	Positive Control	0.406±.04574		

Table 6: Unpaired t-test for comparison of sucrose aided biofilm eradication of A. lebbeck extracts

Extract concen-tration		Mean	t-value	p-value
10 mg	Ethanol	0.349±.124	-7,655	< 0.001 HS
	Ethyl acetate	0.153±.024		
	Hexane	0.014±.004		
	Chloro-form	0.002±.0		
	Positive Control	0.748±.248		
5 mg	Ethanol	0.004±.0	-1,555	< 0.001 HS
	Ethyl acetate	0.003±.0		
	Hexane	0.002±.0		
	Chloro-form	0.004±.0		
	Positive control	0.748±.248		
2 mg	Ethanol	0.004±.0	-45.006	< 0.001 HS
	Ethyl acetate	0.003±.0		
	Hexane	0.067±.006		
	Chloro-form	0.004±.0		
	Positive control	0.748±.248		
.02mg	Ethanol	0.004±.0	-44.1304	< 0.01 HS
	Ethyl ac-etate	0.003±.0		
	Hexane	0.069±.0		
	Chloro-form	0.004±.0		
	Positive control	0.748±.248		

and to subsequently calculate inhibition. Contents of all wells, including the controls were aspirated to reveal the remnant biofilms, which were subjected to MTT assay and culture on validation plates. The least concentration that gave no growth on validation plates was taken as the MBEC.

g. Effect on Sucrose-dependent and Sucrose-independent Adherence to Smooth Glass Surfaces

The effect of the sub-MIC concentrations of the extracts on adherence of *S. mutans* was to a smooth glass surface was evaluated as per Hamada S, et al 1978. The bacteria are grown at 37±2°C for 24 hours, in a glass tube containing 10 ml of BHI with or without 5% (w/v) sucrose and sub-MIC concentrations of the extracts,at an angle of 30^0. The solvents were also incubated under similar conditions as controls. After incubation, the glass tubes were slightly rotated and the planktonic cells decanted. The adhered cells were removed by adding 0.5M Sodium Hydroxide and agitating. The cells were then washed and suspended in saline. The adherence was quantified spectrophotometrically at 600 nm. All tests were performed in triplicates, using untreated BHI medium as control.

Table 7: Mann Whitney U test for the effect of A. lebbeck extracts on the sucrose independent adherence of S.mutans glass surfaces

Adherence without sucrose	Mean	Standard deviation	Median	Z	Significance
Ethanol 10mg	.32667	.025166	0.330	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Ethanol 5mg	.28500	.015000	0.285	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Ethanol 2mg	.25900	.004583	0.260	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Ethanol 0.02mg	.17767	.002082	0.177	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Ethyl acetate 10mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.39033	.007572	0.387		
Ethyl acetate 5mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.39033	.007572	0.387		
Ethyl acetate 2mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.39033	.007572	0.387		
Ethyl acetate 0.02mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.39033	.007572	0.387		
Hexane 10mg	.14667	.003055	0.146	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Hexane 5mg	.12767	.005686	0.126	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Hexane 2mg	.11233	.002309	0.111	-1.993	0.046(S)
Control	.39033	.007572	0.387		
Hexane 0.02mg	.07733	.003786	0.079	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Chloroform 10mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.39033	.007572	0.387		
Chloroform 5mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.39033	.007572	0.387		
Chloroform 2mg	.07200	.004583	0.071	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		
Chloroform 0.02mg	.05067	.009292	0.055	-1.964	0.050(N.S)
Control	.39033	.007572	0.387		

h. Effect on Cell-surface Hydrophobicity of S. mutans

The effect of the extracts on cell surface hydrophobicity of *S. mutans* was measured according to the Microbial adhesion test to hydrocarbon (Martin A, et al 1989).(12)The organism grown in BHI medium, supplemented with 5% sucrose,along with different concentrations of the extracts,were washed twice and suspended in sterile saline (0.85%) so that their optical density (O.D.) is 0.3 at 600 nm. To 3 ml of this cell suspension 0.25 ml of toluene was added, agitated uniformly in a vortex mixer for 2 min and allowed to equilibrate at room temperature for 10 min. Following the toluene and aqueous phase separation the O.D. of the aqueous phase was determined spectrophotometrically at 600 nm. A value of <70% was considered hydrophobic.

i. Effect on Acid Production

The effect of different concentrations of the plant extracts on acid production of the *S. mutans* was assessed according to *Ciardi JF, et al* 1981 (13). 5 ml of BHI broth containing 5% (w/v) of sucrose and the sub-MIC concentrations of the extracts were inoculated with 100 µl of 24-hour cultures of *S. mutans* and adjusted on the McFarland scale to obtain a final inoculum of 1.5 X 104 CFU/ml and incubated at 37±2°C for 24 hours. The pH of the bacterial broth was assessed at 0 and 24 hours after incubation. All determinations were performed in triplicates using necessary controls.

Table 8: Mann Whitney test for the effect of A. lebbeck extracts on the sucrose dependent adherence of S.mutans glass surfaces

Adherence with sucrose	Mean	Standard deviation	Median	Z	Significance
Ethanol 10mg	.00000	.000000	0	-2.087	0.037(S)
Control	.42367	.026633	0.417		
Ethanol 5mg	.37167	.011719	0.367	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Ethanol 2mg	.36000	.005000	0.360	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Ethanol 0.02mg	.19467	.006110	0.196	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Ethyl acetate 10mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.42367	.026633	0.417		
Ethyl acetate 5mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.42367	.026633	0.417		
Ethyl acetate 2mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.42367	.026633	0.417		
Ethyl acetate 0.02mg	.00000	.000000	0.000	-2.087	0.037(S)
Control	.42367	.026633	0.417		
Hexane 10mg	.16133	.005033	0.162	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Hexane 5mg	.16267	.005774	0.166	-1.993	0.046(S)
Control	.42367	.026633	0.417		
Hexane 2mg	.13533	.007506	0.131	-1.993	0.046(S)
Control	.42367	.026633	0.417		
Hexane 0.02mg	.09700	.003606	0.098	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Chloroform 10mg	.08933	.001528	0.089	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Chloroform 5mg	.08700	.004000	0.087	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Chloroform 2mg	.04233	.008145	0.046	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		
Chloroform 0.02mg	.03567	.006429	0.033	-1.964	0.050(N.S)
Control	.42367	.026633	0.417		

j. Effect on Growth in the presence and absence of sucrose

The 24-hour culture of organisms in BHI was adjusted to the McFarland standard 0.5 to yield a cell density of 1.5x 108 CFUs/ml and 50 µl was dispensed in each well of a sterile microtiter plate along with 50 µl of the extracts at different sub-MIC concentrations in the presence and absence of 5% sucrose and incubated at 37±2°C for 24 hours. 100µl of McFar-land 0.5 standard adjusted inoculum was used as a positive growth control and plain broth and solvents were the negative controls. The plates were incubated at 37 ± 2^{0}C for 24 hours and turbidity then read at 600 nm. All determinations were performed as triplicates.

Table 9: One-way Anova for analysing the effect of the growth of various A.lebbeck extracts on S.mutans in absence of sucrose

Growth without Sucrose	Mean	Standard deviation	F	Significance
Ethanol 10mg	.167889	.0661895	46.900	.000 (H.S)
Ethanol 5mg	.135278	.0312350		
Ethanol 2mg	.101722	.0127593		
Ethanol 0.02mg	.114500	.0369917		
Control	.275556	.0496129		
Ethyl acetate 10mg	.061111	.0411681	103.097	.000 (H.S)
Ethyl acetate 5mg	.054611	.0435257		
Ethyl acetate 2mg	.040722	.0377231		
Ethyl acetate 0.02mg	.035167	.0408559		
Control	.275556	.0496129		
Hexane 10mg	.091611	.0342948	61.288	.000 (H.S)
Hexane 5mg	.079667	.0387374		
Hexane 2mg	.048000	.0369133		
Hexane 0.02mg	.130000	.0719043		
Control	.275556	.0496129		
Chloroform 10mg	.096000	.0134164	113.390	.000 (H.S)
Chloroform 5mg	.113722	.0150911		
Chloroform 2mg	.149444	.0249381		
Chloroform 0.02mg	.182222	.0224724		
Control	.275556	.0496129		

Results

Phytochemical analysis and antioxidant properties

The results of the phytochemical analysis are shown in Table 1.

a. Alkaloids were found in the chloroform extracts; phenolics only in the ethanolic extracts; flavonoids in the chloroform, ethyl acetate and hexane extracts; proteins only in ethanolic extracts and terpenoids were found only in the chloroform extracts.

The ethyl acetate extract showed the highest antioxidant capacity (76.97 mg/gm ascorbic acid equivalent) followed by the ethanolic extract (63.11 mg/g ascorbic acid equivalent). Hexane and ether extracts showed the least antioxidant activity (75.3mg/gm ascorbic acid equivalent).

b. Determination of MIC

Among all the extracts tested only the ethyl acetate extract (Table 2, Fig 1) showed zones of inhibition at concentrations of 100,75,50 and 25 mg/ml, none of the other extracts tested showed any zone of inhibition, however the ability of the extracts to diffuse into the agar remained questionable. None of the plain solvents inhibited the growth of the organisms. Hence the MIC was determined at 25mg/ml and all other tests were performed at the sub-MIC concentrations of 10mg/ml, 5mg/ml, 2mg/ml and 0.02mg/ml.

c. Biofilm Inhibition

The effect of the various extracts on the biofilm formation (Figure 2) of *S. mutans* and their statistical comparison using the Mann Whitney U is shown in tables 3 and 4. The control group (*S.mutans* in BHI) predictably showed the highest formation of biofilms with a reading of 0.37367±0.02. Among the test groups all concentrations of the ethanolic and ethyl acetate extracts showed a statistically significant ($p<0.05$) inhibitory effect on the ability of *S.mutans* to form biofilms with total elimination of biofilm formation.

The addition of 5% sucrose to the growth mixture, as expected, increased the capability of *S.mutans* to form biofilms. However, this did not in any major way alter the ability of the extracts to inhibit biofilm formation with the ethanolic extracts (other than the 5mg/ml) and the ethyl acetate extracts demonstrating significant inhibition. One notable exception is that the chloroform extracts did not significantly inhibit the ability of biofilm formation in the presence of Sucrose.

d. Biofilm Eradication

All concentrations of extracts eradicated pre-formed *S.mutans* biofilms as demonstrated by the MTT assay (Graph 1). The MTT values when subject to a t-test (Table 5) show a statistically significant ability to eradicate pre-formed biofilms in the absence of sucrose with all concentrations tested showing highly significant eradication capabilities. Similar results were obtained for biofilms formed in the presence of sucrose also (Table 6, Graph 2).

Table 10: Tukeys Post-hoc test for analyzing the effect of the growth of various A.lebbeck extracts on S.mutans in absence of sucrose

Growth in the absence of sucrose			Mean difference	Standard error	Significance	95% Confidence Interval	
						Lower bound	Upper bound
Ethanol	10mg	5mg	.0326111	.0144140	.167(N.S)	-.007563	.072786
		2mg	.0661667	.0144140	.000(H.S)	.025992	.106341
		0.02mg	.0533889	.0144140	.003(H.S)	.013214	.093563
		Control	-.1076667	.0144140	.000(H.S)	-.147841	-.067492
	5mg	2mg	.0335556	.0144140	.146(N.S)	-.006619	.073730
		0.02mg	.0207778	.0144140	.603(N.S)	-.019397	.060952
		Control	-.1402778	.0144140	.000(H.S)	-.180452	-.100103
	2mg	0.02mg	-.0127778	.0144140	.901(N.S)	-.052952	.027397
		Control	-.1738333	.0144140	.000(H.S)	-.214008	-.133659
	0.02mg	Control	-.1610556	.0144140	.000(H.S)	-.201230	-.120881
Ethyl acetate	10mg	5mg	.0065000	.0142540	.991(N.S)	-.033229	.046229
		2mg	.0203889	.0142540	.610(N.S)	-.019340	.060118
		0.02mg	.0259444	.0142540	.369(N.S)	-.013784	.065673
		Control	-.2144444	.0142540	.000(H.S)	-.254173	-.174716
	5mg	2mg	.0138889	.0142540	.866(N.S)	-.025840	.053618
		0.02mg	.0194444	.0142540	.652(N.S)	-.020284	.059173
		Control	-.2209444	.0142540	.000(H.S)	-.260673	-.181216
	2mg	0.02mg	.0055556	.0142540	.995(N.S)	-.034173	.045284
		Control	-.2348333	.0142540	.000(H.S)	-.274562	-.195105
	0.02mg	Control	-.2403889	.0142540	.000(H.S)	-.280118	-.200660
Hexane	10mg	5mg	.0119444	.0161045	.946(N.S)	-.032942	.056831
		2mg	.0436111	.0161045	.061(N.S)	-.001275	.088498
		0.02mg	-.0383889	.0161045	.130(N.S)	-.083275	.006498
		Control	-.1839444	.0161045	.000(H.S)	-.228831	-.139058
	5mg	2mg	.0316667	.0161045	.291(N.S)	-.013220	.076553
		0.02mg	-.0503333	.0161045	.020(S)	-.095220	-.005447
		Control	-.1958889	.0161045	.000(H.S)	-.240775	-.151002
	2mg	0.02mg	-.0820000	.0161045	.000(H.S)	-.126886	-.037114
		Control	-.2275556	.0161045	.000(H.S)	-.272442	-.182669
	0.02mg	Control	-.1455556	.0161045	.000(H.S)	-.190442	-.100669
Chloroform	10mg	5mg	-.0177222	.0094235	.336(N.S)	-.043987	.008543
		2mg	-.0534444	.0094235	.000(H.S)	-.079710	-.027179
		0.02mg	-.0862222	.0094235	.000(H.S)	-.112487	-.059957
		Control	-.1795556	.0094235	.000(H.S)	-.205821	-.153290
	5mg	2mg	-.0357222	.0094235	.003(H.S)	-.061987	-.009457
		0.02mg	-.0685000	.0094235	.000(H.S)	-.094765	-.042235
		Control	-.1618333	.0094235	.000(H.S)	-.188098	-.135568
	2mg	0.02mg	-.0327778	.0094235	.007(H.S)	-.059043	-.006513
		Control	-.1261111	.0094235	.000(H.S)	-.152376	-.099846
	0.02mg	Control	-.0933333	.0094235	.000(H.S)	-.119598	-.067068

e. Sucrose-dependent and Sucrose-independent Adherence to Smooth Glass Surfaces

The ethanolic extract at 10mg/ml, all concentrations of the ethyl acetate extract, and 5mg/ml and 2mg/ml concentrations of the hexane extract inhibited the sucrose-dependent adherence properties of *S.mutans* (Table 8). The sucrose independent adherence properties of *S.mutans* were inhibited by all extracts of ethyl acetate, 2mg/ml of hexane, and 10 & 5 mg/ml extracts of chloroform (Table 9). None of the other tested concentrations of extracts showed significant inhibition.

Table 11: One-way Anova for analyzing the effect of the growth of various A. lebbeck extracts on S.mutans in presence of sucrose

	Mean	Standard deviation	F	Significance
Ethanol 10mg	.202056	.0475487	55.425	0.000 (H.S)
Ethanol 5mg	.317944	.0803042		
Ethanol 2mg	.201056	.0774008		
Ethanol 0.02mg	.087500	.0173248		
Control	.388333	.0838942		
Ethyl acetate 10mg	.033667	.0331219	185.992	0.000(H.S)
Ethyl acetate 5mg	.047333	.0337987		
Ethyl acetate 2mg	.047389	.0332480		
Ethyl acetate 0.02mg	.037500	.0357528		
Control	.388333	.0838942		
Hexane 10mg	.165833	.0555340	38.972	0.000(H.S)
Hexane 5mg	.151333	.0314904		
Hexane 2mg	.171500	.0705618		
Hexane 0.02mg	.192667	.0797880		
Control	.388333	.0838942		
Chloroform 10mg	.190000	.0579198	21.718	0.000(H.S)
Chloroform 5mg	.164222	.0662025		
Chloroform 2mg	.214278	.0867917		
Chloroform 0.02mg	.197778	.1055014		
Control	.388333	.0838942		

f. Effect on Cell-surface Hydrophobicity of S. mutans

The influence of the various extracts on the cell surface hydrophobicity of *S.mutans* is depicted in Graph 3. The ethanolic extracts at 10 & 5 mg/ml, all concentrations of ethyl acetate and hexane extracts were successful in inducing hydrophobicity. None of the chloroform extracts and solvents tested showed these capabilities

g. Effect on Acid Production

When S.mutans aliquots were incubated in the presence of various extracts and pH was measured post 24 hours incubation, there was no significant pH drop in all the ethanolic and ethyl acetatetest wells.The hexane and chloroform extracts and solvents though, were not effective in inhibiting *S.mutans* acid production and showed a pH drop similar to the control as shown in Graph 4.

The addition of 5 % sucrose potentiated the acidogenicity of the S.mutans aliquots after 24 hours incubation. The ethanolic and ethyl acetate extracts were able to inhibit acidogenicity even in the presence of sucrose. Hexane and chloroform extracts and showed a pH drop almost similar to the control as shown in Graph 5.

h. Effect on Sucrose-dependent and Sucrose-independent Planktonic Growth

Similar to the tests done on the biofilms, we also tested the capability of the various *A. lebbeck* extracts to inhibit the growth of planktonic forms of S.mutans in the presence and absence of sucrose. The results of the turbidometric assay when subjected to a one-way ANOVA and post-hoc Tukey showed that all extracts tested were able to significantly inhibit the growth of S.mutans in comparison to the control ($p<0.05$) and the ethyl acetate extracts showed a dose-independent inhibition of growth (Table 9). The addition of 5% sucrose did not influence the capabilities of the extracts in inhibiting the growth of S.mutans as seen in Tables 10 and 11.

Discussion

Dental caries is a common multifactorial disease occurring in the oral cavity and is widely prevalent.[14] The microbial involvement in this disease especially of the mutans streptococci, specifically *S.mutans* has been well researched and established. The traditional methods of caries control, while largely effective, have their shortcomings and hence the necessity to develop alternatives against this microorganism.[4] The field of ethnomedicine and phytochemicals has numerous plants which have been used as antimicrobials in traditional medicine. There is a perceptive trend worldwide towards medical products sourced from ethnomedical sources.[15] There exists an increasing body of evidence in literature focusing on various plant extracts with effective antimicrobial action against S.mutans while their role inhibiting its other virulence factors have largely been ignored.[16]

This study is focused on the effect of various extracts of *A.lebbeck* on the virulence factors of *S.mutans* that lead to dental caries. With traditional methods of antimicrobial testing- well diffusion method- Only the ethyl acetate extract of A.lebbeck exhibited antibacterial action at a MIC of 25mg/ml (12.5±1.6mm inhibition zone). Similar results were found at a concentration

Table 12: Tukeys Post hoc test for analyzing the effect of the growth of various A. lebbeck extracts on S.mutans in presence of sucrose

			Mean difference	Standard error	Significance	95% Confidence Interval	
						Lower bound	Upper bound
Ethanol	10mg	5mg	-.1158889	.0221304	.000(H.S)	-.177571	-.054207
		2mg	.0010000	.0221304	1.000(N.S)	-.060682	.062682
		0.02mg	.1145556	.0221304	.000(H.S)	.052874	.176237
		Control	-.1862778	.0221304	.000(H.S)	-.247960	-.124596
	5mg	2mg	.1168889	.0221304	.000(H.S)	.055207	.178571
		0.02mg	.2304444	.0221304	.000(H.S)	.168763	.292126
		Control	-.0703889	.0221304	.017(S)	-.132071	-.008707
	2mg	0.02mg	.1135556	.0221304	.000(H.S)	.051874	.175237
		Control	-.1872778	.0221304	.000(H.S)	-.248960	-.125596
	0.02mg	Control	-.3008333	.0221304	.000(H.S)	-.362515	-.239152
Ethyl acetate	10mg	5mg	-.0136667	.0160979	.914(N.S)	-.058535	.031201
		2mg	-.0137222	.0160979	.913(N.S)	-.058590	.031146
		0.02mg	-.0038333	.0160979	.999(N.S)	-.048701	.041035
		Control	-.3546667	.0160979	.000(H.S)	-.399535	-.309799
	5mg	2mg	-.0000556	.0160979	1.000(N.S)	-.044923	.044812
		0.02mg	.0098333	.0160979	.973(N.S)	-.035035	.054701
		Control	-.3410000	.0160979	.000(H.S)	-.385868	-.296132
	2mg	0.02mg	.0098889	.0160979	.972(N.S)	-.034979	.054757
		Control	-.3409444	.0160979	.000(H.S)	-.385812	-.296077
	0.02mg	Control	-.3508333	.0160979	.000(H.S)	-.395701	-.305965
Hexane	10mg	5mg	.0145000	.0223403	.966(N.S)	-.047767	.076767
		2mg	-.0056667	.0223403	.999(N.S)	-.067934	.056600
		0.02mg	-.0268333	.0223403	.751(N.S)	-.089100	.035434
		Control	-.2225000	.0223403	.000(H.S)	-.284767	-.160233
	5mg	2mg	-.0201667	.0223403	.895(N.S)	-.082434	.042100
		0.02mg	-.0413333	.0223403	.352(N.S)	-.103600	.020934
		Control	-.2370000	.0223403	.000(H.S)	-.299267	-.174733
	2mg	0.02mg	-.0211667	.0223403	.877(N.S)	-.083434	.041100
		Control	-.2168333	.0223403	.000(H.S)	-.279100	-.154566
	0.02mg	Control	-.1956667	.0223403	.000(H.S)	-.257934	-.133400
Chloroform	10mg	5mg	.0257778	.0272597	.878(N.S)	-.050200	.101756
		2mg	-.0242778	.0272597	.900(N.S)	-.100256	.051700
		0.02mg	-.0077778	.0272597	.999(N.S)	-.083756	.068200
		Control	-.1983333	.0272597	.000(H.S)	-.274311	-.122355
	5mg	2mg	-.0500556	.0272597	.360(N.S)	-.126034	.025923
		0.02mg	-.0335556	.0272597	.733(N.S)	-.109534	.042423
		Control	-.2241111	.0272597	.000(H.S)	-.300089	-.148133
	2mg	0.02mg	.0165000	.0272597	.974(N.S)	-.059478	.092478
		Control	-.1740556	.0272597	.000(H.S)	-.250034	-.098077
	0.02mg	Control	-.1905556	.0272597	.000(H.S)	-.266534	-.114577

of 48% of the ethyl acetate extract in a recent study with other extracts not demonstrating antibacterial action.[17]

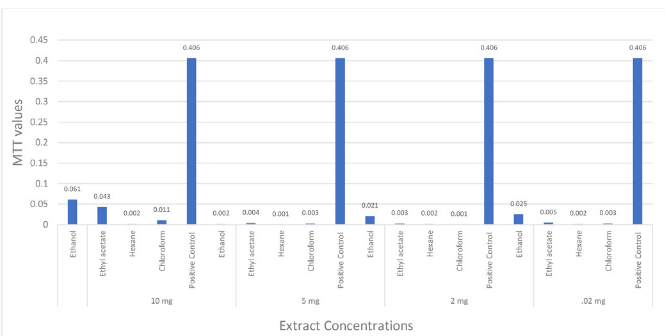
The formation, growth and maintenance of biofilms is an important factor by which *S.mutans* elicits the carious process and hence any agent that can control biofilms can interrupt the carious process.[18] The sub-MIC concentrations of ethanolic and ethyl acetate extracts tested in this study were able to inhibit the formation of *S.mutans* biofilms even at the lowest tested concentrations (Table 3), the addition of sucrose (a potentiator of biofilm formation) did not inhibit the anti-biofilm actions of these extracts (Table 4). All extracts tested also demonstrated an ability to eradicate formed bacterial biofilms (Tables 5,6). Thus,we could establish that extracts which did not demonstrate an antibacterial effect were able to inhibit a different virulence factor i.e., biofilm formation.

S. mutans demonstrates the capability of adherence to tooth surfaces through sucrose dependent independent mechanisms and this property is considered essential for the development of caries.[19] The ethyl acetate extract at all concentrations and 10mg/ml ethanol and 5 and2mg/ml hexane were able to significantly reduce, both the sucrose dependent and independent, adherence of the tested *S.mutans* strain to glass thereby demonstrating an inhibition of the adherence properties of *S.mutans*.

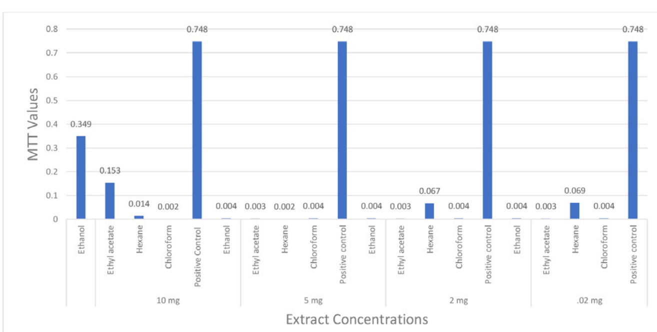
One of the most important factors modulating adherence and thereby biofilm formation of microorganisms is, cell surface hydrophobicity. The more hydrophobic a bacteria is the better they are at forming biofilms and vice versa 19,20 To assess if the anti-biofilm and anti- adherence properties of the extracts were an extension of their antibacterial action the extracts were tested for their action on the cell surface hydrophobicity of S.mutans. All ethyl acetate and hexane extracts and 10, 7 and 5 mg/ml concentrations of the ethanolic extracts were found to decrease the cell surface hydrophobicity of the *S.mutans* strain tested.

Acid production by *S.mutans* in the presence and absence of sucrose was also inhibited by the ethanolic and ethyl acetate extracts. Whether this was a consequence of the antibacterial action or were the extracts inhibiting acid production by any other means is not evident from our study.

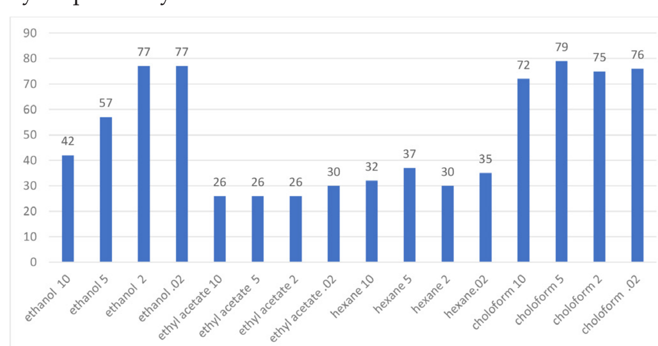
Graph 1: MTT assay of biofilm eradication capabilities of *A.lebbeck* extracts on *S. mutans* biofilms



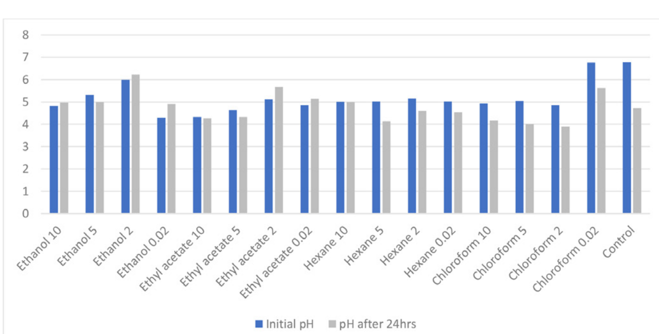
Graph 2: MTT assay of biofilm eradication capabilities of *A.lebbeck* extracts on sucrose aided *S. mutans* biofilms



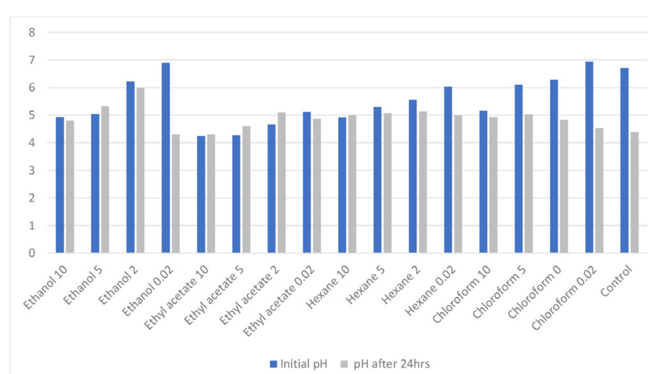
Graph 3: Effect of various extracts of *A. lebbeck* on cell surface hydrophobicity of *S. mutans*



Graph 4: Effect of various extracts of *A. lebbeck* on the acidogenicity of *S. mutans* without sucrose



Graph 5: Effect of various extracts of *A.lebbeck* on the acidogenicity of *S.mutans* with 5% sucrose



The growth of planktonic forms of S.mutans was found to be inhibited by all extracts tested.

Conclusion

The extracts of the A.lebbeck bark demonstrated a potent antibacterial action against the *S.mutans* strain (ATCC 25175). The extracts have also demonstrated inhibitory effects against the various virulence properties of *S.mutans* that aid it in causing dental caries. The fact that these properties are demonstrated at sub-MIC concentrations of crude extracts demonstrates the immense potential this plant harbours to be used in oral healthcare formulation.

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