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Tamarindus indica (Cesalpiniaceae), and *Syzygium cumini* (Myrtaceae) Seed Extracts Can Kill Multidrug Resistant *Streptococcus mutans* in Biofilm

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Abstract

Extracts of *Emblica officinalis* seeds prepared by Microwave Assisted Extraction (MAE) method were evaluated for their antimicrobial property against planktonic form of certain human/plant pathogenic microbes. Additionally, seed extracts of *E. officinalis*, *Tamarindus indica, Manilkara zapota*, *Phoenix sylvestris*, *Syzygium cumini*, and selected phytocompounds were tested against multi-drug resistant *Streptococcus mutans* (a major pathogen associated with human dental caries) in its planktonic as well as biofilm form. Ability of these extracts to eradicate and kill *S. mutans* biofilm was investigated. *E. officinalis* extracts exerted bactericidal action against *S. mutans, Pseudomonas aeruginosa*, and *Vibrio cholerae*. Acetone extract of *S. cumini*, and curcumin were able to inhibit *S. mutans* at appreciably low concentrations of 50 µg/mL and 20 µg/mL respectively. *T. indica and S. cumini* seed extracts were able to kill ≥ 80% cells of *S. mutans* in biofilm, in the concentration range of 500–1000 µg/mL. These extracts were able to achieve ≥ 95% killing of *S. mutans* biofilm at concentrations ranging from 600–2000 µg/mL. Ability of the potent extracts to kill *S. mutans* biofilm did not seem to be much dependent on eradication of the biofilm. Extraction efficiency was found to have a good correlation with antibacterial activity.

Keywords: Antibacterial, Biofilm, Drug-resistance, Microwave Assisted Extraction (MAE), Minimum Inhibitory Concentration (MIC), *Emblica officinalis*.

1. Introduction

Emergence of drug resistant pathogens has been a major concern in the field of medical microbiology. Implication of multidrug resistant microbial pathogens in hospital infections makes it necessary to search for novel antimicrobials. In the US alone, the extra cost for treating the drug resistant variety is around $10 billion per year [1]. Plant products being different in structure than those of microbial origin, may exert antimicrobial activity by a novel mode of action. Seeds being the primary stage of plant life cycle have strong defence mechanism. The presence of bioactive substances (including antimicrobial compounds) in plant seeds have been reported extensively [2, 3]. Screening the crude plant extracts for their desired bioactivity is among the most important operations in medicinal plant research.

It is much more difficult to kill microorganisms in biofilm, as compared to their planktonic counterpart. Microbial biofilms are known to be much more resistant/tolerant to antimicrobials than the planktonic form of the same species. When cells exist in a biofilm, they can become 10–1000 times more resistant to the effects of

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antimicrobial agents [4, 5]. Biofilms have been associated with wide range of problems in industry, in medicine (dental plaque, clinical infections), and in agriculture. *Streptococcus mutans* is a major pathogen associated with human dental caries [6]. Among its important virulence properties is the ability to form biofilms (dental plaque) on tooth surfaces [7]. Eradication of microbial biofilms is a difficult task. Conventional antibiotics effective against planktonic pathogens may not prove equally useful against biofilms. Efforts are being made to find antimicrobial substances effective against biofilms, from plant extracts [8, 9].

The present study aimed at screening *Emblica officinalis* Gaertn. (*Phyllanthus emblica* Linn.) seed extracts against planktonic form of few human/plant pathogenic microbes for their antimicrobial property. Additionally *S. mutans* in its planktonic as well as biofilm form was challenged with seed extracts of *E. officinalis* (Euphorbiaceae), *Tamarindus indica* (Leguminosae), *Manilkara zapota* (Sapotaceae), *Phoenix sylvestris* (*Phoenix dectylifera*; Arecaceae), and *Syzygium cumini* (*Eugenia jambolana;* Myrtaceae). Ability of these extracts to eradicate and kill *S. mutans* biofilm was investigated.

2. Materials and Methods

2.1 Plant Materials

Seeds of all the five plants, *E. officinalis, T. indica, S. cumini, P. sylvestris*, and *M. zapota* were procured during October, 2012 to February, 2013, from the fruits purchased from local market in the city of Ahmedabad. They were authenticated for their unambiguous identity by Dr. Himanshu Pandya, Department of Botany, Gujarat University, Ahmedabad.

2.2 Test Organisms

Following test organisms (Table 1) were procured from Microbial Type Culture Collection (MTCC), Chandigarh.

2.3 Extraction

Seeds were extracted in three different solvents (Merck, Mumbai, India) - acetone, methanol, and ethanol (50%) by microwave assisted extraction (MAE) method [10]. One gram of dry seed powder was soaked into 50 mL of solvent, and subjected to microwave heating (Electrolux EM30EC90SS) at 720 W. Total heating time was kept

Table 1: Test organisms

No.	Organism	MTCC Code	[a]Remarks (with input from MTCC catalogue)
1	*Streptococcus mutans*	497	Isolated form carious dentine, Resistant to streptomycin (up to 30 µg/mL), cefaclor and cefotaxime.
2	*Streptococcus pyogenes*	442	Resistant to gentamicin, and streptomycin tolerant (up to 15 µg/mL)
3	*Staphylococcus aureus*	737	Recommended for antibiotic sterility testing
4	*Staphylococcus epidermidis*	435	Isolated form skin lesion, streptomycin tolerant (upto 10µg/mL)
5	*Pseudomonas aeruginosa*	2474	Isolated from ONGC campus, Dehradun
6	*Vibrio cholerae*	3906	-
7	*Salmonella paratyphi A*	735	Tolerant to streptomycin (10 µg/mL)
8	*Escherichia coli*	1687	Isolated from feces
9	*Xanthomonas campestris*	2286	Originally collected from cabbage leaves bearing black rot; resistant to cefaclor
10	*Pectobacterium caratovorum*	1428	Collected from potato; Resistant to streptomycin, ampicillin, penicillin, cefuroxime, cotrimoxazole, cefacolor, cefafroxil, and amikacin
11	*Pseudomonas syringae*	673	Isolated from soil of penguin rookery with algae; resistant to cefaclor, cefafroxil, and ampicillin
12	*Candida albicans*	3017	Resistant to amphotericin, and fluconazole tolerant (up to 10µg/ml)

[a]antibiotic susceptibility determined by microbroth dilution assay and disc diffusion assay in our lab.

120, 90 and 70 second for acetone, methanol and ethanol, respectively, with intermittent cooling. This was followed by centrifugation (at 7,500 rpm for 20 min), and filtration with Whatman paper # 1 (Whatman International Ltd., Maidstone, England). Solvent was evaporated from the filtered extract and then the dried extracts were reconstituted in dimethyl sulfoxide (DMSO) for antimicrobial assay. Reconstituted extracts were stored under refrigeration for further use. Extraction efficiency was calculated as percentage weight of the starting dried plant material.

2.4 Broth Dilution Assay Against Planktonic Form of Pathogens

All the test organisms were challenged with different concentrations of *E. officinalis* seed extracts. Remaining seed extracts were tested only against *S. mutans*, as their antimicrobial property against other pathogens have already been published by us [11–14]. MIC (minimum inhibitory concentration) was determined using micro-broth dilution method as per NCCLS guidelines [15]. Assay was performed in 96-well microtitre plates (HiMedia TPG96). Total volume of the assay system in each well was kept 200 µL. Muller-Hinton broth (HiMedia) was used as growth medium. As this medium did not support growth of *S. mutans*, experiments involving *S. mutans* were performed in brain heart infusion broth (BHI; HiMedia). Extracts of *S. cumini, T. indica,* and *P. sylvestris* were getting precipitated in organic media, therefore while testing them, minimal media (sucrose 15 g/L, K_2HPO_4 5.0 g/L, NH_4Cl 2 g/L, NaCl 1 g/L, $MgSO_4$ 0.1 g/L, yeast extract 0.1 g/L, pH 7.4±0.2) was used. *C. albicans* was grown in RPMI-1640 (HiMedia) supplemented with glutamic acid (10.3 mg/mL) [16]. Inoculum density of the test organisms was adjusted to that of 0.5 McFarland standard. Broth was dispensed into wells of microtitre plate followed by addition of test extract and inoculum. Extracts (reconstituted in DMSO) were serially diluted into each of the wells. A DMSO control was included in all assays [17]. Gentamicin (HiMedia) served as positive control for bacteria, and fluconazole (HiMedia) for *C. albicans*. Appropriate abiotic controls (containing media and extract, but no inoculum) were also set. In case of human pathogens, plates were incubated at 35°C for 16–20 h, before being read at 655 nm in a plate reader (BIORAD 680). Phytopathogens were incubated at 30˚C for 16–20 h. *S. mutans* in minimal media was incubated for 24 h. Duration of incubation was 48 h in case of *C. albicans*. MIC was recorded as the lowest concentration at which no growth was observed. All MICs were determined on three independent occasions. Concentration at which growth was inhibited by 50% was recorded as IC_{50} value. After reading the plates for MIC, subculturing was made on nutrient agar from the wells showing no growth, so as to determine whether the extract is bactericidal or bacteriostatic. Incubation was continued for 72 h to detect any possible post extract effect (PEE) [14], as the agents exhibiting a post antibacterial effect (PAE)/post antifungal effect (PAFE) require extended incubation following subculture in either time-kill or minimum lethal concentration (MLC) determinations in order to ensure the detection of slow-growing but not dead organisms [18]. Growth on the plate indicated bacteriostatic action, absence of growth was interpreted as bactericidal action, and the concentration at which nearly 99.9% killing was observed was taken as minimum bactericidal concentration (MBC). Delayed growth as compared to control was interpreted as Post Extract Effect (PEE). Total activity (mL/g) was calculated as [19]: Amount extracted from 1 g (mg) / MIC (mg/mL). Activity index was calculated as ratio of MIC of antibiotic to MIC of test extract against susceptible organisms [20].

Pure phytocompounds namely gallic acid (Sisco research laboratories pvt. Ltd., Mumbai), quercetin (s. d.fine-chem Ltd., Mumbai), and curcumin (Central drug house Ltd., New Delhi) were also tested against *S. mutans* using the method described above for extracts.

2.5 Susceptibility Testing Against Biofilm

Extracts which exerted antibacterial activity against planktonic *S. mutans* in broth dilution assay, were also evaluated against its biofilm. Other susceptible organisms could not form good biofilm in the polystyrene plate employed by us.

Standardized inoculum was added into the wells of a 96-well surface treated polystyrene microtiter plate (HiMedia, TPP96), using BHI supplemented with 2% sucrose [8, 21] as the growth medium. Uninoculated autoclaved medium was put in wells corresponding to sterility control. Total volume of the content filled in the wells was kept 300 µL. Incubation to allow biofilm

formation was carried out at 35˚C for 48 h. Following incubation spent medium was removed from the wells under aseptic condition, and wells were filled with minimal media containing test extract (or antibiotic used as positive control). DMSO replaced the extract in the wells corresponding to negative control. Organism was incubated in this medium containing test extract for 24 h at 35˚C. Each concentration of the test extract was put in six replicate wells. Following incubation in media containing test extract, three of these wells were used for assessment of biofilm viability. Remaining three were subjected to crystal violet assay [8, 22] for assessing biofilm eradication.

For the crystal violet assay the biofilm-coated wells of microtitre plates were vigorously shaken in order to remove all non-adherent (planktonic) bacteria. The remaining attached bacteria were washed twice with 200 µL of 50 mmol/L phosphate buffer saline (PBS; pH 7.0) and air-dried for 45 min. Then, each of the washed wells was stained with 110 µL of 0.4% aqueous crystal violet solution for 45 min. Afterwards, each well was washed twice with 200 µL of sterile distilled water and immediately de-stained with 200 µL of 95% ethanol. After 45 min of de-staining, 100 µL of de-staining solution was transferred to a new well and the amount of the crystal violet stain in the de-staining solution was measured with microplate reader (BIO-RAD 680) at 655 nm.

Viability of the biofilm was estimated by two different methods- viable plate count [23], and tube method (suspending the biofilm in liquid media). For plate count, biofilm was removed from the well surface by scraping with a sterile micropipette tip. This scrapped biofilm was added to sterile normal saline, and vortexed for 30 s. Serial 10-fold dilutions in sterile normal saline were prepared from each sample. Measured volumes of each dilution were dispensed onto nutrient agar plates and then incubated upto 72 h at 35°C. *S. mutans* when transferred from biofilm to fresh solid medium, it took longer (48 h) for visible growth to appear, as compared to planktonic cells, which could give rise to visible growth within 24 h. After 48 h of incubation, colonies were counted to estimate the total viable cell counts from each well. Percentage viability was then assessed relative to those of the negative controls. In case of antibiotic treated wells, comparison was made with growth controls (medium plus organism). Incubation was extended till 72 h, at the end of which colony count was made once again to confirm killing of the cells in biofilm.

Viability assessment through tube method employing liquid media was also performed in parallel from same dilutions (which were used for viable plate count experiment described above), to investigate correlation between these two techniques. Briefly, measured volume of the serial dilution was inoculated into defined quantity of sterile nutrient broth, and incubated for 24 h at 35°C. Following incubation growth was quantified in terms of OD at 625 nm (Spectronic 20D+, Thermo scientific). Percentage viability in the tubes corresponding to test wells was calculated in relation to OD of the negative control. Tubes from antibiotic treated wells were compared with the growth control. Concentration at which 80% [15] and 95% killing was achieved, were taken respectively as MIC and MBC against biofilm form. These values are hereafter mentioned in this paper as MIC_{BF} and MBC_{BF}.

3. Results

Results of MAE are reported in Table 2. Results of broth dilution assay of all the extracts against various test strains are presented through Table 3–4. Results obtained while evaluating seed extracts against biofilm are recorded in Table 5–6.

Table 2: Extraction and reconstitution efficiency for all the seed extracts

Seed	Solvent	Extraction efficiency (%)	Reconstitution efficiency (%)
E. officinalis	Ethanol (50%)	5.08	81.58
	Methanol	4.30	69.09
	Acetone	1.66	55.45
T. indica	Ethanol (50%)	15.06	40.85
	Methanol	19.90	87.23
	Acetone	6.76	89.05
S. cumini	Ethanol (50%)	15.28	86.52
	Methanol	10.78	83.42
	Acetone	5.92	98.53
M. zapota	Ethanol (50%)	9.36	86.78
	Methanol	9.27	70.40
	Acetone	7.87	79.67
P. sylvestris	Ethanol (50%)	7.18	67.55
	Methanol	8.60	73.54
	Acetone	6.78	71.97

Table 3: Results of broth dilution assay of *E. officinalis* seed extracts

Solvent used for extraction	Organism	IC_{50}	MIC	MBC	Total activity	Average total activity	[a]Activity index	MBC/MIC
		(µg/mL)			(mL/g)			
Ethanol (50%)	*S. mutans*	–	400	400	127	80.43	0.025	1
	S. pyogenes	400	>1000	PEE	NA		NA	
	S. aureus	100	>1000	BS				
	V. cholerae	1000	1500	1500	33.86		0.066	1
Methanol	*S. pyogenes*	200	>1000	PEE	NA	45.38	NA	
	S. aureus	100	>1000	BS				
	P. aeruginosa	–	900	900	47.77		0.011	1
	V. cholerae	–	1000	BS	43.00		0.010	NA
Acetone	*S. mutans*	–	1000	BS	16.70		0.010	
	S. pyogenes	300	>1000	PEE	NA	22.72	NA	
	S. aureus	200	>1000	BS				
	P. aeruginosa	500	900	900	18.44		0.011	1
	V. cholerae	–	500	BS	33.02		0.020	NA

[a]Activity index was calculated using MIC values obtained with ampicillin, which was 10 µg/mL for all organisms in question; BS- Bacteriostatic; PEE - Post Extract Effect; NA- Not Applicable

Table 4: Results of broth dilution assay of different plant products against *S. mutans*

Seed/ Phytocompound/ Antibiotic	Solvent	MIC	MBC	Total activity	Activity index		MBC/MIC
		(µg/mL)		(mL/g)	A	B	
E. officinalis	Ethanol (50%)	400	400	127	0.100	0.025	1
	Methanol	>1000		NA	NA	NA	NA
	Acetone	1000	BS	16.60	0.040	0.010	
T. indica	Ethanol (50%)	465		323.87	0.086	0.021	
	Methanol	350	350	568.57	0.114	0.028	1
	Acetone	500	500	135.20	0.080	0.020	1
S. cumini	Ethanol (50%)	500	500	305.60	0.080	0.020	1
	Methanol	500	500	215.60	0.080	0.020	1
	Acetone	50	300	1184	0.800	0.200	6
P. sylvestris	Ethanol (50%)	NI	BS	NA	NA	NA	NA
	Methanol	400		215	0.100	0.025	
	Acetone	400		169.50	0.100	0.025	
M. zapota	Ethanol (50%)	>1000			NA	NA	
	Methanol	>1000					
	Acetone	>1000					
Curcumin	Dissolved in DMSO	20			2.000	0.500	
Quercetin		>100		NA	NA	NA	
Gallic acid		>100					
Streptomycin	Dissolved in sterile distilled water	40					
Ampicillin		10	ND				
Gentamicin		10					

A: Activity index calculated using MIC values obtained with streptomycin.
B: Activity index calculated using MIC values obtained with ampicillin.
BS: Bacteriostatic; NA: Not Applicable; ND: Not Done; NI: No Inhibition.

Table 5: Eradication and killing of *S. mutans* biofilm caused by various seed extracts / antibiotics

Plant seed/ Antibiotic	Solvent	Conc. (µg/ml)	% Eradication	% Loss of viability	
				Tube method	Plate count method
T. indica	Methanol	350	NE	NI	NI
		500	NE	NI	NI
		600	NE	89.89±3.52	98.84±0.93
		700	NE	86.31±2.23	99.24±0.54
		800	NE	87.36±2.93	94.11±4.53
		900	NE	89.99±0.74	95.64±4.11
		1000	28.84±2.84	87.70±12.30	87.57±0.00
		1084	55.06±6.23	94.57±5.43	97.75±1.23
		1500	39.85±0.20*	100±0.00	99.48±0.53
		1700	26.29±5.75	86.07±1.36	97.54±2.39
		2000	16.01±6.60*	90.0±10.00*	98.24±1.03
	Acetone	500	NE	NI	NI
		600	20.76±10.57*	75.78±13.33*	82.72±10.92
		800	33.37±12.51	86.95±7.50	99.87±0.00
		900	43.73±2.14	76.56±7.83	96.18±2.86
		1024	36.44±13.92	90.55±9.45	97.13±0.39
		1500	14.67±1.12	69.11±2.60	84.83±1.99
		1800	30.91±4.86*	92.01±1.14	97.57±2.03
S. cumini	Ethanol (50%)	500	NE	NI	NI
		1000	NE	88.71±9.09	97.72±0.07
		1500	NE	87.83±10.21	97.74±1.68
		2000	NE	88.43±11.02	94.66±2.48
		2500	40.18±3.28	81.33±2.40	95.61±0.42
	Methanol	500	NE	80.52±8.76	80.37±4.18
		1000	NE	75.64±18.42	91.25±8.59
		1500	25.35±8.90*	77.54±16.74*	92.98±6.92
		2000	32.62±10.24*	82.28±4.70	99.47±0.52
		2500	40.21±2.31*	61.18±11.38	72.79±15.40*
	Acetone	50	NE	NI	NI
		500	NE	33.26±11.68	41.93±0.00
		1000	NE	76.08±8.08	83.52±5.04
		1500	NE	86.29±13.71	90.58±0.36
		2000	NE	94.15±5.85	98.03±1.97
		2500	38.61±0.18*	93.00±6.93	98.27±1.72
Ampicillin	Dissolved in sterile distilled water	10	NE	NI	NI
		20	NE	NI	NI
		30	NE	NI	NI
		40	NE	92.48±2.19	97.81±1.81
		50	37.65±1.14*	94.92±5.07	99.43±0.56
Gentamicin	Dissolved in sterile distilled water	10	NE	92.34±7.65	99.95±0.00
		20	NE	100±0.00	99.95±0.00
		30	NE	100±0.00	99.93±0.09
		50	18.73±0.57*	100±0.00*	99.18±0.81
		100	19.70±3.47	95.52±2.52*	99.96±0.02

All values are significant with $p<0.01$, except those marked with *; *$p<0.05$;
NE: No Eradication; NI: No Inhibition

Table 6: Comparison between effect of various plant extract on planktonic and biofilm form of *S. mutans*

Plant seed/ Antibiotic	Solvent	Planktonic		Biofilm		Total activity (mL/g) (Biofilm)	[a]Activity index (Biofilm)	MIC_{BF}/ MIC_P
		MIC_P	MBC_P	MIC_{BF}	MBC_{BF}			
		(µg/ml)						
E. officinalis	Ethanol (50%)	400	400	>800	>800	NA	NA	>2
T. indica	Methanol	350	350	600	600	331.6	0.066	1.71
	Acetone	500	500	600	800	112.6	0.066	1.20
S. cumini	Ethanol (50%)	500	500	1000	1000	152.8	0.040	2
	Methanol	500	500	500	2000	215.6	0.080	1
	Acetone	50	300	1000	2000	59.2	0.020	20
Ampicillin	Dissolved in	10	10	40	40		NA	4
Gentamicin	sterile distilled water	10	10	10	10			1

[a]Activity index calculated using MIC value obtained with ampicillin; NA: Not Applicable; MBC_{BF}: Conc. required to achieve ≥95% killing of biofilm; MIC_{BF}: MIC against biofilm; MIC_P: MIC against planktonic cells; MBC_P: MBC against planktonic cells.

4. Discussion

4.1 Extraction

Extraction and reconstitution efficiency of all the seeds in different solvents is recorded in Table 2. Highest extraction efficiency (19.9%) was obtained in case of methanolic extract of *T. indica* seeds, followed by hydroalcoholic extract of *S. cumini* seeds. With respect to extraction yield, ethanol and methanol proved better than acetone for all seeds. During MAE, presence of water in the extracting solvent is believed to facilitate better heating of the plant matrix, as water can penetrate easily into the plant cells. Using water in combination with ethanol or any other solvent in MAE can increase the mass transfer of the active constituents into the extracting fluid [24]. However while reconstituting the dried extracts of *S. cumini* and *T. indica* in DMSO, higher reconstitution efficiency was obtained with their acetone extracts. It should be noted that the possible advantage(s) of high extraction yield may be somewhat compromised by low reconstitution efficiency, because some of the phytoconstituents present in the original extract may be left out due to inability of the reconstituting solvent to solubilize all of them.

4.2 Broth Dilution Assay

Among all the extracts of *E. officinalis*, its ethanolic extract was most effective against *S. mutans* (Table 3), with bactericidal effect on it. This extract registered a total activity of 127 mL/g against *S. mutans*, which means that if 1 g of this extract is diluted in 127 mL of the solvent, still it would be able to inhibit *S. mutans*. Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. It indicates the degree to which the active fractions or compounds present in 1 g can be diluted and still inhibit growth of the test organism [19]. For *E. officinalis* seed extracts, the *average total activity* was found to have a positive linear correlation (r= 0.90) with the *extraction efficiency*. Strong correlation between these two quantities has also been reported earlier [11, 25]. Acetone extract of *E. officinalis* was effective against three different gram-positive and gram-negative bacteria at ≤ 1 mg/mL concentration, with a bactericidal action against *P. aeruginosa*. This extract exerted a broad spectrum of antibacterial activity, and was most effective against *V. cholarae*. Importance of finding new anti-vibrio compounds is evident from the fact that in parts of India, the main drugs used to treat cholera (furazolidone, ampicillin) have gone from being highly effective to essentially useless in 10 years [1]. Methanol extract of *E. officinalis* also proved bactericidal against *P. aeruginosa*. In all cases of bactericidal activity of *E. officinalis* seed extracts, the MBC/MIC ratio was 1. A small MBC/ MIC ratio (< 4 to 6) is usually expected for bactericidal agents [26]. This ratio between 1 to 2 has been suggested as indicator of bactericidal mode of action [27, 28, 29]. Importance of bactericidal action of extracts against *P. aeruginosa* is evident from the fact

that this pathogenic bacterium is notoriously infamous for its multidrug resistance and involvement in nosocomial infections. A number of efflux pumps have been described in it, which confer resistance to multiple antibiotics including fluoroquinolone and penicillins [30]. Finding bactericidal agents against gram-negative bacteria is of particular value, as they are generally much harder to find "hits" against, presumably as a result of their outer membrane in cell wall which greatly decreases permeability, and because they are intrinsically resistant through the expression of membrane bound efflux pumps [31]. All the three extracts of *E. officinalis* exhibited a PEE effect against *S. pyogenes*, whose duration was 48 h in all three cases i.e., the extracts significantly, reduced ability of *S. pyogenes* to revive on a extract free nutrient medium. Visible growth on nutrient agar (after plating from wells containing inhibitory concentration of the test extract) was obtained only after 48 h of incubation (it took 24 h when contents were plated from control well). Similar effect for antibiotics termed as Post Antibacterial Effect (PAE)/ Post Antifungal Effect (PAFE) has been described in literature [18]. PEE with seed extracts of *P. sylvestris, T. indica*, and *S. cumini* against pathogenic bacteria and fungi has earlier been reported by us [13,14]. Activity of potent extracts against susceptible microbes was compared to that of ampicillin, and activity index (AI) was calculated. Ethanolic extract of *E. officinalis* registered an AI of 0.025 against *S. mutans*. The more the value of AI, more potent the extract is.

E. officinalis seed extracts were also tested against organisms not listed in Table 3, but mentioned in the list of test organisms (Table 1). However these extracts were not notably effective against those organisms upto 1 mg/mL. Aneja *et al.* (2010) reported *E. officinalis* fruit extract to be effective against *S. mutans* and *S. aureus*, albeit at relatively higher concentrations (12.5–50 mg/mL) [32]. Gupta *et al.* (2012) reported methanolic extract of *E. officinalis* seed to be effective against *P. aeruginosa* at 25 mg/mL (MIC) and 200 mg/mL (MBC) [33]. Extracts of same seed reported in present study are effective against this organism at much lesser concentration of 900 μg/mL (Table 3). Though the extraction yield obtained by them (12.3%) is higher than that obtained by us (1.66–5.08%), antibacterial efficacy is better in our extracts. This heavy variation in activity may be attributed to the difference in methods used for extraction. The present study employed MAE as against Soxhlet method employed by Gupta *et al.* (2012) [33]. Better performance of MAE in extracting antibacterial compounds from plant seeds has previously been reported by us [34]. Increased heat exposure during Soxhlet extraction may result in degradation of plant compounds responsible for anti-pseudomonas activity. MAE is believed to be suitable for effective extraction of heat-labile phytoconstituents [24], which may be destroyed while preparing extracts through other heat-employing methods.

In addition to *E. officinalis, S. mutans* was also challenged with seed extracts of four other plants and certain phytochemicals (curcumin, quercetin, and gallic acid) in pure form (Table 4). Among all the plant products tested, *S. mutans* was most susceptible to curcumin (MIC: 20 μg/mL), followed by acetone extract of *S. cumini* seed (MIC: 50 μg/mL). Latter exerted the highest total activity (1184 mL/g) against *S. mutans*, followed by the methanolic extract of *T. indica* seeds. Among seed extracts highest AI was registered by acetone extract of *S. cumini*, however its MBC/MIC ratio was higher than all other bactericidal extracts. Out of a total of fifteen test extracts, six exerted bactericidal action against *S. mutans*, and MBC/MIC ratio for all of them was 1, except for acetone extract of *S. cumini*. Due to its low MIC, curcumin was able to register a good score of AI (Table 4). It was twice as effective as streptomycin, however the effect was bacteriostatic. Curcumin, a dietary polyphenolic compound, has been known to possess bacteriostatic activity against pathogens like *Aeromonas hydrophila,* and a potent antibacterial activity against a number of pathogenic bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus* [35]. Rais *et al.* (2008) suggested that curcumin inhibits bacterial cytokinesis, and that it strongly inhibited the formation of the cytokinetic Z-ring in *B. subtilis* [36]. Their results indicated that the perturbation of the GTPase activity of FtsZ assembly is lethal to bacteria. Curcumin bioconjugates have been reported to possess antibacterial activity [37]. Curcumin has also been reported as a promising antifungal [38], as well antiprotozoal [39] agent. Use of curcumin as an antimicrobial finish owing to its bactericidal properties on dyed textiles was reported by Hana and Yang (2005) [40]. Curcumin was reported to attenuate the virulence of *Pseudomonas aeruginosa* by inhibiting the virulence factors such as biofilm formation, pyocyanin biosynthesis,

elastase/protease activity, and acyl homoserine lactone production [41]. Curcumin is known for its ability to bind a variety of proteins and inhibit the activity of various kinases. It is believed to be safe even when consumed at a daily dose of 12 g for 3 months [42].

Quercetin and gallic acid have been known as constituents of many plant parts including *S. cumini* seeds [43], and *E. officinalis* [44]. Though two of *E. officinalis* seed extracts, and all the *S. cumini* seed extracts used in this study were effective against *S. mutans*, quercetin and gallic acid were not able to effectively inhibit this organism at the tested concentration. This suggests that phytoconstituents other than quercetin and gallic acid are likely to be more responsible for activity of these extracts against *S. mutans*. Except *M. zapota*, one or more extracts of all other seeds were effective against *S. mutans*. In our previous studies, extracts from *S. cumini* and *P. sylvestris* were found ineffective against *S. pyogenes* [25]. *E. officinalis* seed extracts were effective against *S. mutans*, but not against *S. pyogenes* (Table 3). Although all the three *E. officinalis* extracts failed to completely inhibit *S. pyogenes* upto a concentration of 1 mg/mL, they could inhibit growth of this organism by 50% with IC_{50} values ranging from 200–400 µg/mL.

Among extracts used in this study, those effective against *S. mutans* registered MIC values in the range of 50–1000 µg/mL. Almeida *et al.* (2008) reported *Rheedia brasiliensis* fruit (bacupari) extracts to be effective against *S. mutans*, with MIC values 12.5–25 µg/mL [45]. Bioactive compound (7-epiclusianone) isolated from the crude extract registered lesser MIC (1.25–2.5 µg/mL). Islam *et al.* (2012) reported *T. indica* and *P. emblica* extracts to be effective against *S. mutans* at much higher MIC values (6.25 mg/mL) [46]. Larsen *et al.* (1996) reported paprika and rosemary leaves extracts to be inhibitory to *S. mutans* at MIQ (minimum inhibitory quantity) of 24–180 mg/mL [47]. Jebashree *et al.* (2011) found *Psidium guajava* and *Terminalia chebula* extracts to be effective against *S. mutans* at MIC values ranging from less than 0.076 mg/mL to more than 5 mg/mL [48]. Bacteriostatic activity of guaijaverin, isolated from *Psidium guajava* Linn. leaves against *S. mutans* strains with MIC values of 2–4 mg/mL was reported by Prabu *et al.* (2006) [49]. Islam *et al.* (2008) found *Morus alba* leaf extracts to be effective against *S. mutans* at 125 mg/L, the purified compound (1-deoxynojirimycin) from this extract had an MIC of 15.6 mg/L [50].

Seed extracts which showed bactericidal activity against planktonic form of *S. mutans*, were tested against its biofilm form too. None of other test organisms susceptible to seed extracts were able to form good biofilm on surface treated polystyrene plates used by us. Different extracts were tested at concentrations starting from their MIC against planktonic form (MIC_P) of *S. mutans* to 2500 µg/mL. Except ethanolic extract of *E. officinalis*, all the extracts used against *S. mutans* were able to kill it in biofilm (Table 5–6). Highest total activity (331.6 mL/g) against *S. mutans* biofilm was registered by methanolic extract of *T. indica* seeds, whereas against planktonic form of *S. mutans* acetone extract of *S. cumini* had the highest total activity (Table 4). Interestingly the latter extract had the lowest total activity against *S. mutans* biofilm. Total activity of the potent extracts against *S. mutans* biofilm was found to have a positive linear correlation (r=0.85) with the extraction efficiency, as was the case with planktonic cells. Except methanolic extract of *S. cumini* seeds, all the seed extracts had a MIC_{BF}/MIC_P ratio more than 1, suggesting their reduced efficacy against biofilm form (Table 6). Reduced susceptibility of biofilms to antimicrobials may be attributed to their slow growth rates, high population densities (on the order of 10^{10} cells per mL of hydrated biofilm), and the diffusional barrier posed by the biofilm matrix (De Beer and Stoodly, 2006) Four out of five potent extracts had a MIC_{BF}/MIC_P ratio lesser than that of ampicillin, while gentamicin was effective at same concentration against planktonic as well as biofilm form of *S. mutans*. With respect to ampicillin, highest activity index (0.08) was recorded for methanolic extract of *S. cumini*.

Biofilm eradication caused by the potent extracts was in all cases lesser than viability loss caused by them (Table 5). No notable linear correlation (r=0.30) was found between percent eradication and viability loss of biofilm (revealed through plate count) caused by test extracts. This may be due to the possibility of penetration of biofilm matrix by these extracts without distorting its architecture too heavily. This is more likely to happen for small molecules, or those which do not get adsorbed by the polysaccharide matrix of biofilm. Diffusion of small molecules is not strongly inhibited by the biofilm matrix, whereas diffusion of large molecules is impeded [51]. Hatch and Schiller (1998) showed that alginate lyase allowed more effective diffusion of gentamicin and

tobramycin through alginate, the biofilm polysaccharide of *P. aeruginosa* [52]. Antimicrobial molecules must diffuse through the biofilm matrix in order to inactivate the encased cells. The extracellular polymeric substances constituting this matrix present a diffusional barrier for these molecules by influencing either the rate of transport of the molecule to the biofilm interior, or the reaction of the antimicrobial material with the matrix material [53]. Antimicrobials like the extracts reported in present study, if can overcome the diffusion barrier posed by biofilm matrix, without necessarily breaking it, then may kill the cells inside the biofilm without eradicating it completely. The extent to which the matrix acts as a barrier to drug diffusion would depend on the chemical nature of both the antimicrobial preparation and the matrix material [54].

Methanolic extract of *S. cumini* registered identical MIC values (500 µg/mL) against both planktonic and biofilm form of *S. mutans*, however its MBC against biofilm form was four times higher than that against planktonic form. Seed extracts reported in this study were able to kill ≥ 80% cells of *S. mutans* in biofilm, in the concentration range of 500–1000 µg/mL. These extracts were able to achieve ≥ 95% killing of *S. mutans* biofilm at concentrations ranging from 600–2000 µg/mL. Though reports are available in literature describing activity of plant products or other antimicrobials against *S. mutans* biofilm, most of them are not able to achieve complete killing of the biofilm, and majority of reports describe inhibition of biofilm formation rather than killing of formed biofilm. The MIC of methanol extracts of *Salvadora persica* (miswak) against cariogenic *S. mutans* was reported to be 2.6 mg/mL by Al-Sohaibani and Murugan (2012) [55]. Rukayadi and Hwang (2006) reported a concentration of 50 µmol/L of xanthorrhizol (XTZ) purified from the rhizome of *Curcuma xanthorrhiz*a Roxb. to be capable of removing 76% of *S. mutans* biofilm [8]. Reduction in biofilm formation by *S. mutans* exposed to subinhibitory concentration of *Dodonaea viscose* var. *angustifolia* extract was reported by Naidoo *et al.* (2012) [56]. Triclosan (0.07 mmol/L) was reported to kill approximately 40% of multi-species biofilm of oral bacteria [57]. Chorhexidine gluconate was reported to kill nearly 40% of multi-species bacterial biofilm at 2.23 mmol/L [58]. Realistic comparison of efficacy of various antimicrobials against *S. mutans* biofilm (in fact, biofilm of any organism) is difficult on account of inherent heterogeneity of microbial biofilms, difference in the nature of antimicrobials and their concentrations employed in different studies.

S. mutans strain used in this study was resistant (in its planktonic form) to cephalosporins (cefaclor, cefotaxime), and streptomycin (upto 30 µg/mL). Ampicillin had a four times higher MIC against biofilm form of *S. mutans* than that against its planktonic form. Gentamicin was able to inhibit both forms of *S. mutans* at identical concentration (10 µg/mL) (Table 5). This may be due to difference in mode of action of these antibiotics. As ampicillin exerts its effect by inhibiting cell wall synthesis [1, 59] it is more effective against the actively growing planktonic cell population in which active cell wall synthesis is likely to happen. Cells in biofilm grow at slower pace [53, 54], and as cell wall synthesis in them may not occur at that high level, they can be less susceptible to antibiotics like ampicillin. Gentamicin works by inhibiting protein synthesis [1, 59], which is occurring even in already grown cells of biofilm, even if they divide at a slow pace. Higher MIC of an antibiotic against biofilms may also be due to their incomplete penetration into biofilm matrix. Gram-positive organisms growing as biofilms are believed to be particularly resistant to a wide variety of antibiotics, as compared to their planktonic counterparts [60]. Protection from antimicrobial agents in biofilms may be based on a mechanism of *persister* cells or phenotypic variant formation. Cells can revert from the persister state when exposed to the growth substrate. Persisters are suggested to accumulate under condition of slow growth, typical of biofilm [61].

In this study two different methods, tube method in liquid media and viable count on agar plate, were used for determining viability of biofilm following antimicrobial challenge. A good correlation (r=0.93) was found between the results of these two methods. However, viable count can always be the method of choice because it is based on the assumption that each living cell will give rise to a single colony [59]. In case of inoculation of extract-treated biofilm into liquid media, the newly generated cells can generate their own daughter cells, making the measured value of OD that much higher, while in case of viable count on solid

media, even the newly formed daughter cells are likely to remain in the same colony rather than forming a new colony, thus final colony count is not likely to be affected. That is how the percent loss of viability (i.e. the magnitude of killing) obtained by tube method are almost always bit lesser than those obtained by viable count (Table 5).

Finding plant products active against cariogenic microbes like *S. mutans* is important as even some commercially available dentifrice products are also reported not to be effective against their biofilms. Listerine® mouth wash, *Cymbopongon citrates* (lemon grass), *Plectrantusamboinicus* (mexican mint), and *Conyzabonariensis* (hairy fleabane) tinctures were reported to possess no inhibitory action against the dental biofilm-forming bacteria [62]. Not so appreciable antimicrobial activity in Listerine®, while complete lack of antimicrobial activity in Toss-K and Senquel-AD against four different dental caries pathogens (including *S. mutans* strain employed in present study) was reported by Aneja *et al.* (2010) [63]. Although several antiplaque agents are available in the market, the search for more effective agent(s) still continues [49]. Plant products effective against oral pathogens like *S. mutans* can find use as ingredients in chewing sticks, toothpastes or other dentifrice products. Fractionation of the potent extracts identified in this study can further yield the active principle(s) with still lower MIC than the crude parent extracts. Structural studies of such active principles can pave way for identification of novel lead compounds.

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