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Assessment of antimicrobial properties of *Terminalia chebula* (fruit) against cariogenic organisms

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ABSTRACT

The present study was designated to evaluate the antimicrobial activities of various solvent extracts from fruits of Terminalia chebula. The preliminary antimicrobial activities of the extracts against cariogenic organisms were tested by using disc diffusion assay. The methanolic extract showed higher antimicrobial activity against all the cariogenic organisms tested. Based on this finding, the methanolic fruit extract of T. chebula was assessed for antimicrobial activities by disc diffusion, broth dilution and biofilm inhibition methods. The highest activity was at 400µg/ml of methanolic extract with a mean diameter of inhibition zone being 29.5mm and a minimum inhibitory concentration (MIC) of ≤0.17mg/ml against S. mutans MTCC 497. The anti-adherence activities of methanolic fruit extract towards the cariogenic organisms were different between the organisms. The maximum percentage of inhibition (88%) was observed for S. mutans MTCC 497 and minimum percentage of (17.2%) inhibition was noticed for S. cerevisiae MTCC 170. The results indicated that the methanolic fruit extract of T. chebula posses antimicrobial properties and so it can be used as a potential source of anti- microbial agents.

Keywords: *T. chebula*, cariogenic organisms, antimicrobial activity, disc diffusion, MIC, anti-biofilm activity.

INTRODUCTION

Dental caries is a common oral bacterial pathology caused by a biofilm consisting of microorganisms present on the tooth surface [1]. Antibiotics such as penicillin and erythromycin have been reported to effectively prevent dental caries in animals and humans but they are never used clinically because of many adverse effects [2]. Therefore, recently the use of herbal mouth washes is increasing [3]. The antimicrobial activities of plant extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [4, 5]. *Terminalia chebula* (TC), belonging to family Combretaceae and commonly known as “Black Myrobalan” is found in India as well as in many Asian countries. *T. chebula* is called the "king of medicines" and is always listed first in the Ayurvedic meteria medica because of its extraordinary powers of healing [6, 7].

There are several assays that can be used to determine antimicrobial activity in plant extracts, including agar diffusion and microplate assays (serial dilution assay). However, the diffusion method is not suitable for testing non-

polar samples or samples that do not easily diffuse into the agar [8]. In the liquid dilution method, turbidity is often taken as an indication of growth, so where the sample is inactive against the microorganism tested, the liquid will appear turbid [9]. The biofilm forming bacteria are resistant to antimicrobial agents due to the lack of penetration of antimicrobial agents [10]. Therefore, it is imperative that efficacy of any extract be evaluated additionally on its ability to inhibit multi-species biofilms which are dynamic and possess variability of flora [11]. Hence, this study was designed to assess the effectiveness of *T. chebula* fruit towards the growth inhibition of gram positive and gram negative bacteria associated with dental caries by disc diffusion, broth dilution and biofilm inhibition methods.

MATERIALS AND METHODS

Collection of important cariogenic organisms

The strains *Enterobacter hormaechei* strain A1, *Enterobacter* sp.A2(2016), *Micrococcus luteus* strain A3, *Klebsiella pneumoniae* strain A4, *Exiguobacterium* sp.A5(2016), *Staphylococcus sciuri* strain A6, *Acinetobacter radioresistens* strain A7, *Weissella confusa* strain A8, *Bacillus cereus* strain A9 and *Bacillus subtilis* strain A10 were previously isolated [12] and were subcultured periodically in nutrient agar slants. The other dental caries causing bacteria *Staphylococcus aureus* MTCC 740, *Enterococcus feacalis* MTCC 439, *Pseudomonas aeruginosa* MTCC 424, *Streptococcus mutans* MTCC 497, *Lactobacillus acidophilus* MTCC 10307 and yeasts *Candida albicans* MTCC 227 *Saccharomyces cerevisiae* MTCC 170, were procured from Microbial Type Culture Collection, IMTECH, Chandigarh and were subcultured on the specific media recommended by them.

Collection and preparation of crude extracts

The dry fruits of *T. chebula* (Combretaceae) were collected from local drug store, Nagercoil, Kanyakumari District. The dry fruits were processed by following the methods of Ncube *et al*. [13] and the methanol, hexane, ethyl acetate and chloroform extracts were prepared as described by Alade and Idobi [14] with minor modifications.

Preparation of bacterial inoculums

The inoculums were adjusted according to 0.5McFarland standard which was prepared by adding 0.05ml of barium chloride ($BaCl_2$) (1.17% w/v $BaCl_2.2H_2O$) to 9.95ml of 0.18M H_2SO_4 (1.0% w/v) with constant stirring. The overnight cultured inoculums of test strains was adjusted to 1.5 x 10^8 CFU/ml equal to that of the 0.5McFarland standard by adding sterile distilled water.

Testing for antibacterial activity of various solvent extracts

The preliminary antimicrobial sensitivity pattern of all the solvent extracts used was determined by the standard disc diffusion method as described by Baur *et al*. [15]. Petriplates were prepared with 20ml mueller hinton agar (beef infusion, 300g/L; casein acid hydrolysate, 17.5g/L; starch, 1.5g/L; agar, 17g/L) for all cariogenic organisms. A lawn of test cultures was prepared by evenly spreading 100µl inoculums (1.5 x 10^8 CFU/ml) with the help of a sterilized spreader onto the entire surface of agar plate. The plates were allowed to dry for 10 minutes before applying the disc. On each plate, discs (6mm) with various solvent extract (100µg) and negative control disc were placed and incubated at 35±2ºC for 24 hrs. After incubation, the antimicrobial activity was evaluated by observing the inhibitory zone around the discs impregnated with plant extracts. The test was repeated 3 times for accurate screening. The best active solvent extract was selected for further study.

Disc diffusion assay for selected plant extract at various concentrations

The cariogenic organisms (100µl of 24hrs suspension, 1.5x10^8 CFU/ml) were swabbed on the top of the sterile mueller hinton agar plates and allowed to dry for 10 minutes. On each plate, selected plant extract discs with varying concentrations (100-400µg) and negative control disc (methanol) were placed within 15 minutes of inoculation of organism and the plates were incubated at 35±2ºC for 24 hrs. After incubation, the antimicrobial activity was evaluated by measuring the diameter of inhibitory zone around the discs impregnated with plant extracts. The test was repeated for three times.

Determination of minimum inhibitory concentration

The method of Yadav *et al.* [16] was used to determine the MIC of selected plant extract against cariogenic organisms with some modifications. The assay was initiated by pouring sterile mueller hinton broth aliquots (100µl) into wells of microtitre plates. Exactly 100 µl of 100mg/ml selected plant extract prepared in dimethyl sulphoxide (DMSO) was taken by micropipette and added into first well of microtitre plate containing100µl mueller hinton broth and mixed using a micropipette. From the first well 100µl of broth and plant extract was transferred to the

second well by twofold dilution method. Similarly 100µl broth and extract were transferred from second well up to seventh well to get concentrations of 10.0, 5.0, 2.5, 1.25, 0.625 and 0.17mgs respectively. The last well (8^{th} well) was considered as positive control (containing 100µl chlorhexidine instead of plant extract). Concentrated suspensions of microorganisms (10µl) were added in each well. The microtitre plates were sealed in a plastic bag with a plastic film sealer before incubating at 37ºC in a incubator for 18hrs. After incubation, 40µl of 0.2mg/ml INT (*p*-iodonitrotetrazolium violet) was added to each well and plates were incubated under dark for 30minutes before observation. The development of red colour, resulting from the formation of red/purple formazan, was indicative of growth (positive indicator of cell viability). MIC values were regarded as the lowest concentrations of the extracts that inhibit the growth of the test organisms (decrease in the intensity of the red formazan colour). The experiments were performed in triplicate.

Determination of biofilm inhibitory activity

150ml of BHI broth (calf brain, infusion 200g/L; beef heart infusion 250g/L; protease peptone 10g/L; dextrose 2g/L; sodium chloride 5g/L; disodium phosphate 2.5g/L) and 20ml of SD broth containing 1% D-glucose were prepared and 5ml of each broth was transferred into respective 30ml screw cap tubes. The broth was then sterilized by autoclaving at 121ºC, 15lbs for 15 minutes. After cooling, the respective tubes were inoculated with 50µl overnight cultures of cariogenic organisms. Then the plant extract of *T. chebula* was added at each tube to give desired concentration (250µg) of extract from stock concentration of 100mg/ml of dimethyl sulphoxide. The tubes were tilted at an angle of 30º and incubated at 37ºC for 18hours. After incubation, the supernatant (non-adherent cells) were carefully decanted without disturbing the adhering cells. The change in medium pH was also noted with the help of the pH meter. The tubes containing biofilm were washed with saline (0.85% NaCl). Then, 3ml of saline was added to each tube and mixed well to separate the cells which adhered the glass surface and optical density (O.D) was recorded at 550nm [16]. The percentage of inhibition was calculated using the following formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control-Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

RESULTS

Preliminary screening of antimicrobial activity of plant extracts

The result of the preliminary antibacterial activity which is used to screen the active plant extract for future study was furnished in Table 1. On overall considerations, the antimicrobial activity of the methanolic fruit extract of *T. chebula* was higher as compared to those of other extracts tested. Hence, the methanolic extract of *T. chebula* was selected for further study.

Table 1 Preliminary antibacterial activity of different extracts of *T. chebula* (fruit) on cariogenic organisms

SL. No	Cariogenic organisms	Methanol	Hexane	Ethyl acetate	Chloroform
1	*E. hormaechei* strain A1	+	-	-	-
2	*Enterobacter* sp. A2(2016)	+	-	-	-
3	*M. luteus* strain A3	+	-	+	-
4	*K. pneumoniae* strain A4	+	-	-	-
5	*Exiguobacterium* sp. A5 (2016)	+	-	+	-
6	*S. sciuri* strain A6	+	-	+	-
7	*A.radioresistens* strain A7	+	-	+	-
8	*W. confusa* strain A8	+	-	+	-
9	*B.cereus* strain A9	+	-	-	-
10	*B. subtilis* strain A10	+	-	-	-
12	*S. aureus* MTCC 740	+	+	+	-
11	*P. aeruginosa* MTCC 424	+	-	+	-
13	*E. feacalis* MTCC 439	+	-	+	-
14	*S. mutans* MTCC 497	+	+	-	-
15	*L. acidophilus* MTCC 10307	+	-	+	-
16	*C.albicans* MTCC 227	+	-	+	-
17	*S. cerevisiae* MTCC170	+	-	-	-

+ zone detected, - no zone detected

Disc diffusion assay at various concentrations

In vitro antimicrobial activities of methanolic fruit extract of *T. chebula* against the cariogenic organisms were studied by the disc diffusion method and the results were showed in Figure 1 and Plate 1. The effects of the methanolic fruit extract of *T. chebula* fruit against *E. hormaechei* strain A1, *Enterobacter* sp.A2 (2016), and *K. pneumoniae* strain A4, were generally similar. Maximum inhibitory activity (18.6mm) was observed at the same concentration of 400µg/ml and the least activity was observed at the concentration of 100µg and the antimicrobial activity was recorded as 11.3mm zone of inhibition in diameter. Among two yeast strains tested, the fruit extract displayed higher antimicrobial activity against *C. albicans* MTCC 227 than *S. cerevisiae* MTCC 170. Among all the cariogenic organisms tested, high activity was found on *S. mutans* MTCC 497 with about 29.5mm of inhibition zone at 400µg/ml of methanolic extract. The extract was also effective at 100, 200 and 300µg/ml of concentration in which 20.1, 25.1 and 28.3mm of inhibition zones were recorded respectively.

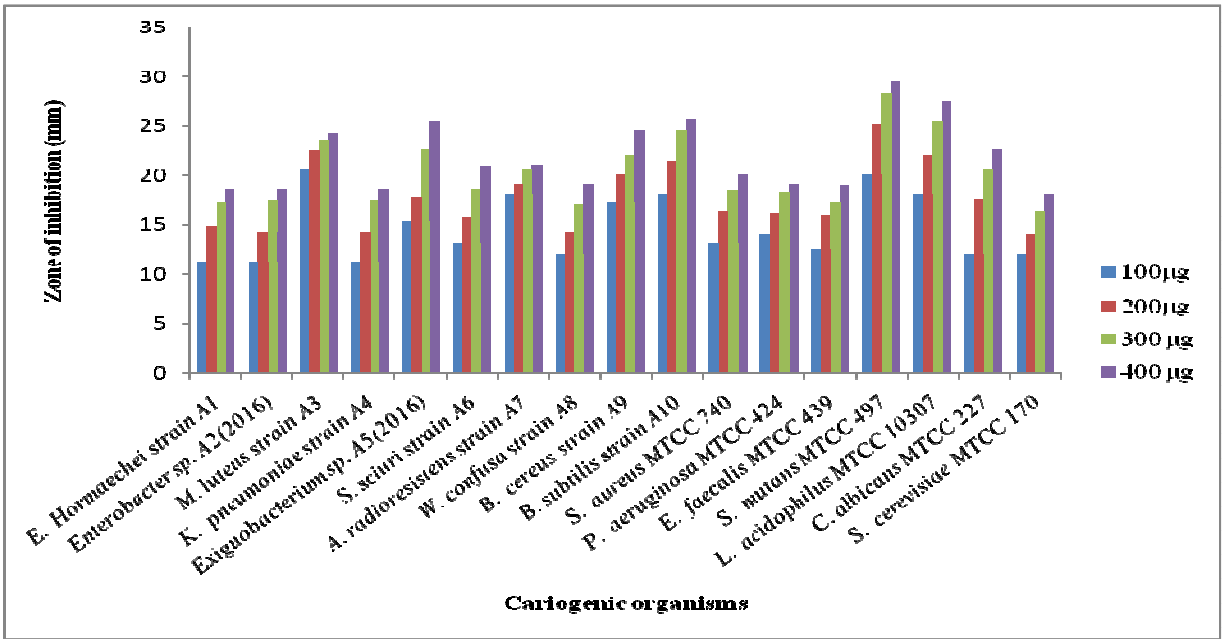

Figure 1 Antimicrobial activity of methanolic fruit extract of *T. chebula* against cariogenic organisms

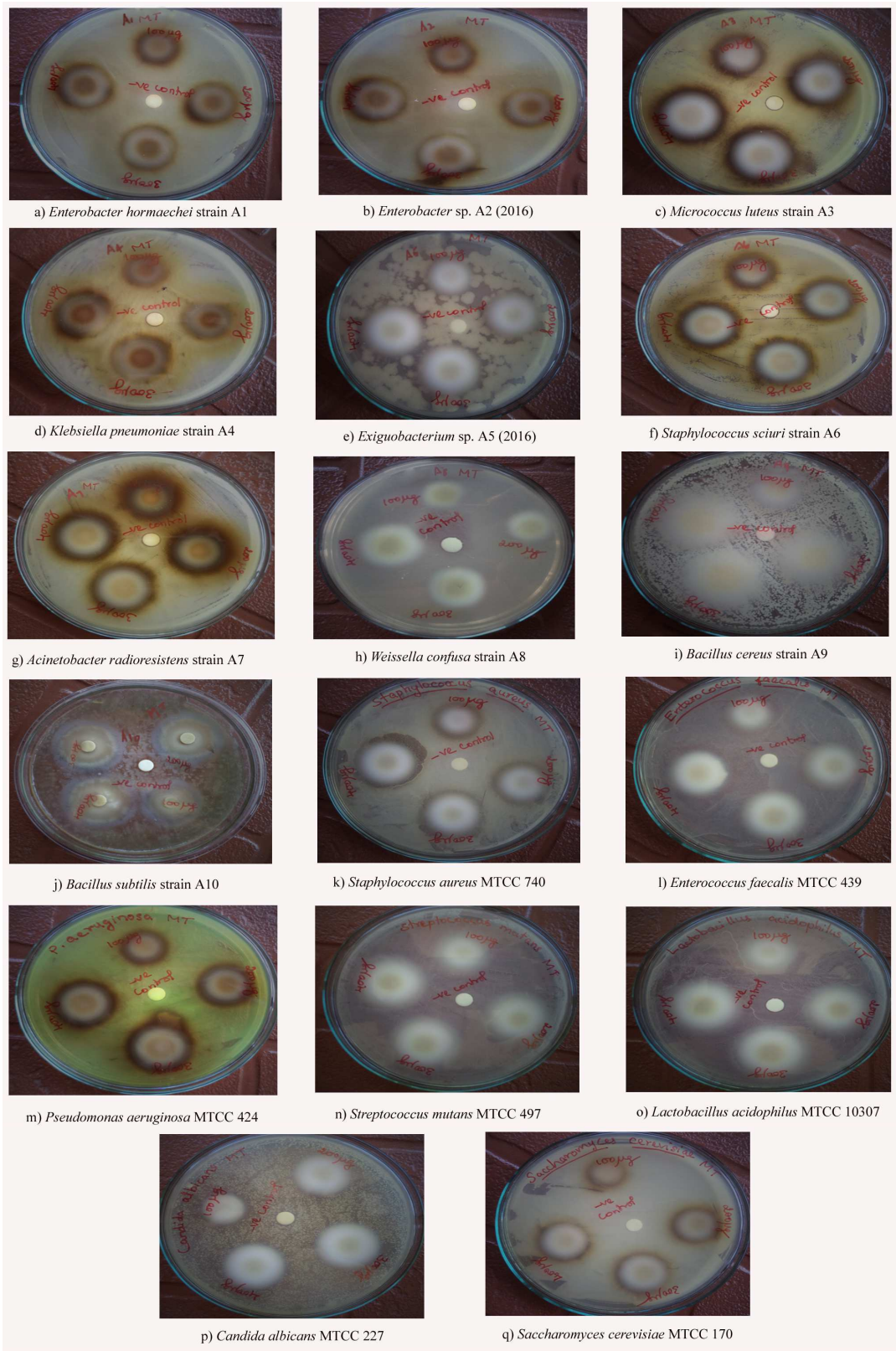

Plate 1 Antimicrobial activity of methanolic fruit extract of *T. chebula* against cariogenic organisms

Determination of MIC for selected plant extract

The quantity of the antibacterial compounds present depends on the MIC values of the extract. Table 2 indicates the minimum inhibitory concentrations of the extracts. The relative growth of the microorganism after 24 hr of incubation in the presence of different concentrations of methanolic fruit extract of *T. chebula* was studied with a positive control chlorhexidine (Plate 2). *S. mutans* MTCC 497 and *L. acidophilus* MTCC 10307 were the most susceptible pathogens that did not survived even at 0.17mg/ml whereas, *K. pneumoniae* strain A4, *S. sciuri* strain A6 and *E. faecalis* MTCC 439 demonstrated the greatest resistance to *T. chebula* (5.0mg/ml) and appeared to be the most resistant bacteria among the strains tested.

Table 2 Minimum Inhibitory Concentration (MIC) of methanol extract of *T. chebula* fruit by broth dilution method

Sl.No	Cariogenic organisms	Methanol extract (mg/ml)
1	*E. hormaechei* strain A1	1.25
2	*Enterobacter* sp. A2(2016)	1.25
3	*M. luteus* strain A3	0.625
4	*K. pneumoniae* strain A4	5.0
5	*Exiguobacterium* sp. A5 (2016)	0.625
6	*S. sciuri* strain A6	5.0
7	*A.radioresistens* strain A7	0.625
8	*W. confusa* strain A8	≤0.17
9	*B.cereus* strain A9	0.625
10	*B. subtilis* strain A10	0.625
11	*S. aureus* MTCC 740	1.25
12	*P. aeruginosa* MTCC 424	1.25
13	*E. feacalis* MTCC 439	5.0
14	*S. mutans* MTCC 497	≤0.17
15	*L. acidophilus* MTCC 10307	≤0.17
16	*C.albicans* MTCC 227	0.31
17	*S. cerevisiae* MTCC170	2.5

≤ = less than or equal to

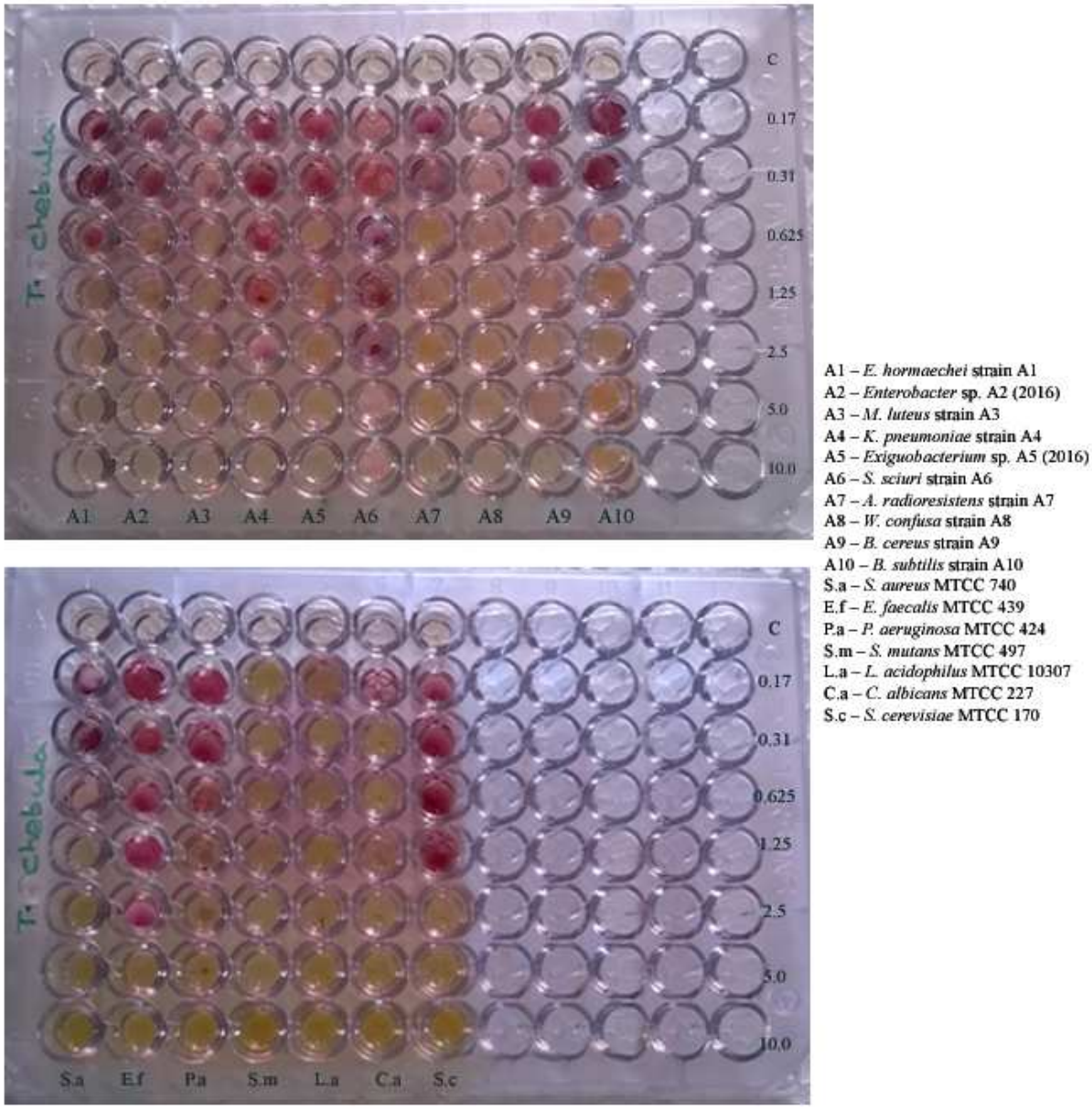

Plate 2 Minimum inhibitory concentration of methanol fruit extract of *T. chebula* against cariogenic organisms

Determination of biofilm inhibitory activity

The methanolic fruit extract of *T. chebula* showed positive antibiofilm effect against organisms on the glass surface of screw cap tube in the presence of 1% glucose. They showed decrease in turbidity when optical density was taken at 550nm (Table 3). The maximum percentage of inhibition (88%) was observed for *S. mutans* MTCC 497 and minimum percentage of (17.2%) inhibition was noticed for *S. cerevisiae* MTCC 170 (Plate 3).

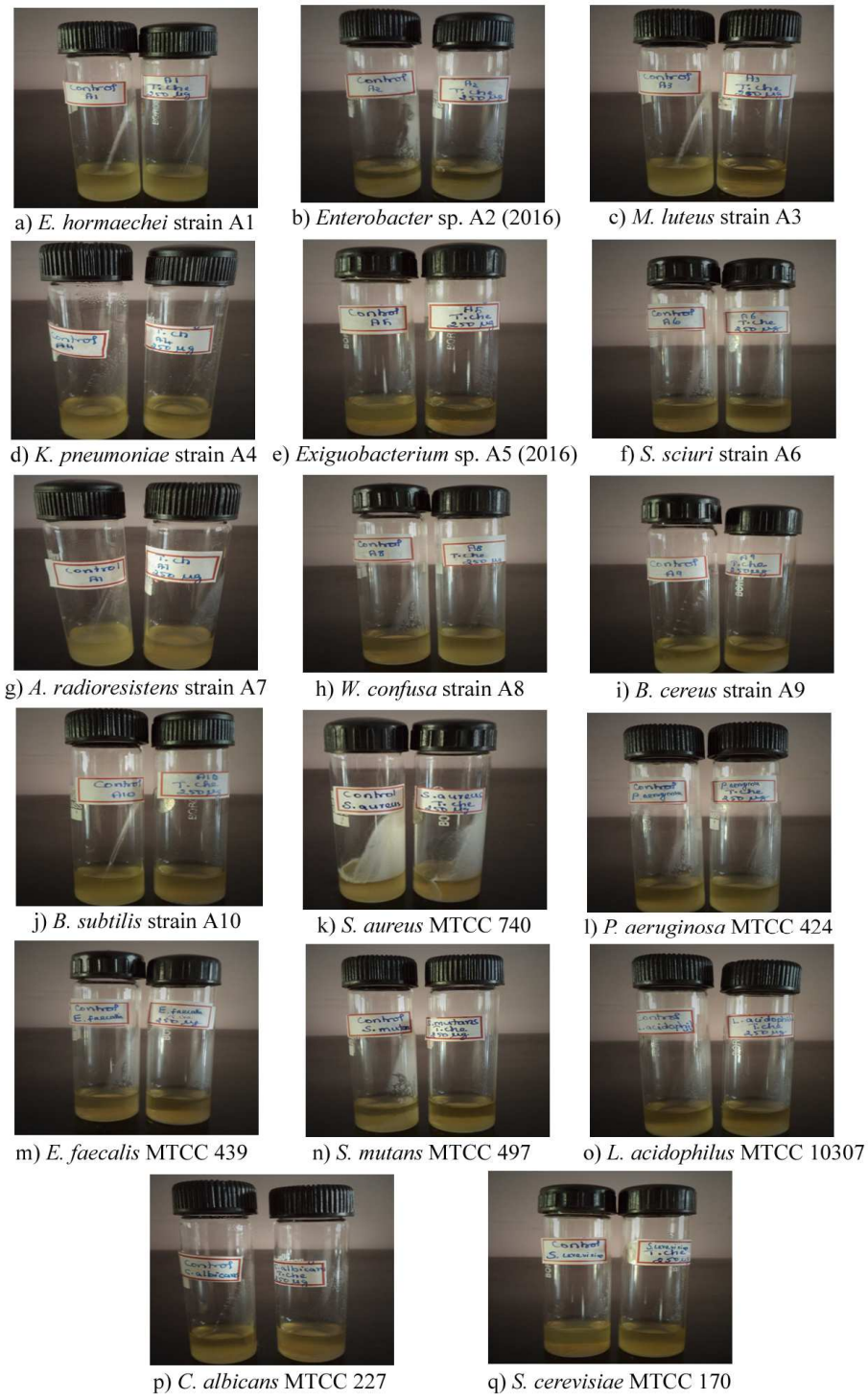

Plate 3 Biofilm inhibitory activity of methanolic fruit extract of *T. chebula* (250µg) against cariogenic organisms

Along with biofilm formation and inhibition, variation of pH in media, between untreated and treated test series were noted and the result was tabulated in Table 3. No variation of pH was noted in the media cultured with untreated and treated *Exiguobacterium* sp. A5 (2016) and *B. cereus* strain A9 and the pH was found to be 7.0 in both

the cases. The variation in pH between treated and untreated media cultured with *S. mutans* MTCC 497 was 2.0. The pH of untreated media was 4.0 and treated media was 6.0 with *S. mutans* MTCC 497.

Table 3 Biofilm inhibitory activity of methanolic fruit extract of *T. chebula* (250µg) on cariogenic organisms and change in pH during activity

Sl.No	Cariogenic organisms	Untreated cells (control)		Treated cells		% of inhibition	Variation in pH
		OD_{550nm}	pH	OD_{550nm}	pH		
1	*E. hormaechei* strain A1	0.685	6.0	0.348	6.7	49.1	0.7
2	*Enterobacter* sp. A2(2016)	0.425	6.1	0.252	6.4	40.7	0.3
3	*M. luteus* strain A3	0.690	6.6	0.112	6.9	83.7	0.3
4	*K. pneumoniae* strain A4	0.760	6.0	0.473	6.5	37.7	0.5
5	*Exiguobacterium* sp. A5 (2016)	0.324	7.0	0.261	7.0	19.4	0.0
6	*S. sciuri* strain A6	0.759	4.7	0.183	6.0	75.8	1.3
7	*A.radioresistens* strain A7	0.634	6.0	0.485	6.3	23.5	0.3
8	*W. confusa* strain A8	0.706	6.2	0.335	6.8	52.5	0.6
9	*B.cereus* strain A9	0.589	6.0	0.085	6.4	85.5	0.4
10	*B. subtilis* strain A10	0.371	7.0	0.070	7.0	81.0	0.0
11	*S. aureus* MTCC 740	0.843	6.5	0.269	6.9	68.3	0.4
12	*P. aeruginosa* MTCC 424	0.751	6.0	0.355	6.4	52.7	0.4
13	*E. feacalis* MTCC 439	0.754	6.1	0.541	6.7	28.2	0.6
14	*S. mutans* MTCC 497	0.862	4.0	0.101	6.0	88.0	2.0
15	*L. acidophilus* MTCC 10307	0.701	4.7	0.204	6.1	70.5	1.4
16	*C.albicans* MTCC 227	0.425	4.5	0.152	6.1	64.0	1.6
17	*S. cerevisiae* MTCC170	0.319	6.6	0.264	7.0	17.2	0.4

DISCUSSION

Antibacterial activities of *T. chebula* against several bacterial strains have been reported by Bag *et al.* [17]; Malekzadeh *et al.* [18]; Kim *et al.* [19] and Chattopadhyay *et al.* [20]. The aqueous and ethanolic extracts of *T. chebula* fruit are known to have antibacterial properties against bacterial isolates *P. aeruginosa, K. pneumoniae, S. sonnei, S. flexneri, S. aureus, V. cholerae, S. paratyphi-B, E. coli, E. faecalis* and *S. typhi* obtained from HIV infected patients [21] and against some pathogens associated with dental caries [22]. *T. chebula* fruit aqueous extracts also have antibacterial activity against methicillin resistant *S. aureus* (MRSA) and trimethoprim-sulphamethoxazole resistant uropathogenic *E. coli* [17]. This study is attempt to strengthen the previous studies to show the therapeutic effect on caries causing bacteria since the extract of *T. chebula* was used as an anticariogenic mouth rinse against three dental caries causing bacteria *S. mutans*, *Lactobacilus* sp. and *Candida* sp. [23]. The result of this study indicated that, the methanol and ethyl acetate extract of *T. chebula* fruit is found to be very effective. Among the four extracts tested, methanol extract inhibited all the 17 strains including 10 gram positive, 5 gram negative bacteria and 2 yeast strains. Since the antimicrobial activity of methanol fruit extract of *T. chebula* was evaluated by the standard disc diffusion and broth dilution method.

Aneja and Joshi [22] reported the highest activity of acetonic fruit extracts of *Terminalia chebula* with a mean diameter of inhibition zone being 25.32mm and a minimum inhibitory concentration (MIC) of 25mg/ml against *S. mutans* and a mean diameter of 32.97mm and MIC of 12.5mg/ml against *S. aureus*. In the present study, methanol extract of *T. chebula* showed maximum activity with zone of inhibition 29.5mm and MIC of ≤0.17mg/ml against *S. mutans* MTCC 497 and mean diameter of 20.1mm and MIC of 1.25mg/ml against *S. aureus* MTCC 740.

Prabhat *et al.* [24] reported that the maximum zone of inhibition against *S. aureus* was 27mm in methanolic extract of *T. chebula.* His study also revealed that the methanolic extracts of *T. chebula* showed maximum antimicrobial activity against *S. mutans* (23mm), *L. acidophilus* (24mm) *S. salivarius* and *C. albicans* (26mm). In the present study, the zone of inhibition of methanol extract of *T. chebula* against *S. mutans* MTCC 497 and *C. albicans* MTCC 227 was 29.5mm and 22.6mm at 400µg/disc concentration. The MIC was found to be ≤0.17mg/ml which was supported by the previous study of Jebashree *et al.* [25] who reported that *T. chebula* showed greater effectiveness against pathogens such as *S. mutans* and *C. albicans.*

It has been previously reported that *T. chebula* can be employed as an effective anti-plaque agent and can be used in the prevention of dental caries [26]. The cariogenic organisms ferment different sugars and they metabolize sugars to lactic acid more rapidly forming a drop in pH which is the main supportive platform for plaque formation [27]. In

the present study, the methanolic fruit extract of *T. chebula* has been tested for its antibiofilm activity against the cariogenic organisms. When the cariogenic organisms were checked for biofilm formation, the media pH drastically decreases from initial neutral pH indicating the change of medium from neutral to acidic. Thus, when the organisms were treated with methanolic fruit extract of *T. chebula*, it not only reduces the biofilm forming ability but also the acidic nature of pH was reduced. Therefore it is evident in this study that *T. chebula* showed a definite reduction in the microbial activity and an increase in the pH resulting in marked anticariogenic effect. Increase in pH was an essential pre-requisite for an ideal mouth rinse. The findings of the present study confirmed the positive relationship with the previous report [28, 29].

This work also showed that the methanol fruit extract of *T. chebula* possessed antimicrobial activity and they can be used as broad spectrum antibiotics since they were active against both gram positive and gram negative bacteria. It is hoped that this study would lead to the establishment of some compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin.

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