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Excellent Antimicrobial and Antibiofilm Effect of Herbal Extracts on Plaque Colonizers and Caries Inhabitants: An *In-Vitro* Study

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Oral health and hygiene are of high concern with respect to human health. Most of the chemical formulation used to control growth of unwanted oral microorganisms and biofilm formation causes several side effects in long term use. Traditional knowledge and herbal extracts may offer better alternatives in addressing oral health issues. This study highlights inhibitory effect of combination of *Emblica officinalis*, *Terminalia belerica*, *Terminalia chebula* (EOTBTC) and *Linum usitatissimum* (LU) extracts on aerobic and anaerobic (facultative and strict) microorganisms and biofilms. EOTBTC and LU extracts were found to inhibit the growth *Streptococcus mutans, Candida albicans, Staphylococcus aureus*, *Lactobacillus acidophillus, Fusobacterium nucleatum* and *Porphyromonas gingivalis.* Zone of Inhibition *(*ZoI) ranged from 3 mm to 36 mm with EOTBTC extract (6 to 36 mg/mL) and with LU extract (6 to 36 mg/mL), ZoI 5 mm to 23 mm. Minimum Inhibitory Concentration MIC of all microorganisms reduced with EOTBTCLU, as compared to individual extract. Reduction in biofilm formation with EOTBTCLU was also noticeable. *C. albicans* and *S. aureus* biofilms were more sensitive to extract (6 mg/mL) while *P. gingivalis* have shown minimum reduction in biofilm at 24 mg/mL concentration. EOTBTCLU extract possessed broad-spectrum antimicrobial and antibiofilm effect and may prove to be better alternative for controlling microorganisms associated with dental caries and biofilms.

Keywords: Biofilm, Broad-spectrum antimicrobial, Minimum Inhibitory Concentration, Traditional knowledge, Trifala

Introduction

Regardless of several remarkable technological advancement and awareness related to dental hygiene eradication of dental caries and associated diseases is not possible.[1] Also, emergence of antibiotic resistance among caries associated microorganism has raised concern related to periodontal problems.[2] Wide variety of microflora exist in oral cavity that have different role to play in oral health of individuals. Disruption in oral cavity microenvironment gives opportunity to opportunistic pathogens present in oral cavity to attack and cause diseases.[3–5] Among these *Streptococcus sp.* (*Streptococcus salivarius*, *S. sanguis*), *Candida* sp. are well known primary plaque colonisers which create favourable anaerobic conditions in plaque to support growth of successive plaque colonisers (strict anaerobes) such as *Fusobacterium* sp. and *Porphyromonas* sp., *Enterococcus faecalis*.[5,6]

Use of herbal preparations is advantageous since they contain more than one bioactive ingredients that may target multiple site in microorganisms and thereby control growth and proliferation of variety of microorganisms. Also, being herbal and biocompatible they do not exert any side effects to individuals.

In view of existing knowledge on phytochemical composition of *Emblica officinalis* (EO), *Terminalia belerica* (TB), *Terminalia chebula* (TC) *extracts* and *Linum usitatissimum* (LU) have attracted attention of authors to study their effect on caries causing microorganisms and report their effect on biofilm formation.[7–9] *Emblica officinalis* (EO), *Terminalia belerica* (TB), *Terminalia chebula* (TC) when combined is traditionally known as Triphala. Although, lot of literature published till date reports Triphala antibacterial activity against oral aerobic microorganism but only limited literature states its effect on secondary and tertiary plaque colonizers.[8,9] Flex seeds being rich in phenolic compound exhibits several bioactive components possessing antimicrobial activity and medicinal properties while their similarity with estrogen creates buffering effect on metabolism of estrogen.[10,11] Although, several researchers have

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reported antimicrobial activity of triphala and its extracts on *Streptococcus mutans*, *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Salmonella typhimurium*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Lactobacillus* sp., *Escherichia coli*, but studies on strict anaerobic bacteria (secondary plaque colonisers) such as *Lactobacillus acidophillus*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are very limited.[12–14] Although, triphala and linseed both contain wide variety of bioactive compounds having antibacterial activity yet studies on antimicrobial spectrum of triphala and linseed combination are not reported.

Present study provides experimental evidence of antibacterial effect of ethanolic extracts of Triphala (EO, TB, TC, LU and EOTBTCLU (combination) extract against primary, secondary and tertiary plaque colonizers i.e., *Streptococcus mutans, Candida albicans, Staphylococcus aureus*, *L. acidophilus, F. nucleatum, P. gingivalis*. EOTBTCLU extract effectiveness in reducing the biofilm formation under *in-vitro* conditions have been assessed in this study.

Materials and Methods

Preparation of Extract

Commercially available EO, TB, TC and LU powder was procured from Pharmacy shop and extracted using cold infusion method. Ten grams of herbal powdered was dissolved in 100 mL of ethanol in a pre-sterilised glass bottle with continuous stirring up to 48 hours at 10°C. The extract obtained was filter sterilized using Whatmann filter paper No. 1. Thereafter, solvent was removed using vacuum concentrator (Eppendrof$^{(R)}$ Centrifugal Concentrator), at 25°C. Dried EO, TB, TC extracts were combined in 1:1:1 ratio (Triphala) and for EOTBTCLU (1:1) EO, TB, TC and LU were mixed in 1:1:1:1 ratio. Extract were appropriately diluted in DMSO (10% v/v) to make different concentrations (6 to 36 mg/mL) of extract to determine Minimum Inhibitory Concentration (MIC) of extract against plaque colonizers. Phytochemical analysis of crude extracts was also performed as per standard protocols.

Procurement of Microorganisms

Lyophilized cultures of *Streptococcus mutans* (ATCC 25175), *Candida albicans* (ATCC 18804), *Staphylococcus aureus* (ATCC 23235) *L. acidophilus* (ATCC 314), *F. nucleatum* (ATCC 10953), *P. gingivalis* (ATCC 33277) were procured from American Type Culture Collection (ATCC), USA for testing the antimicrobial activity. Columbia Blood Agar containing 5% v/v Sheep Blood was used for culturing all test bacteria. *S. mutans, C. albicans, S. aureus* were grown aerobically while *L. acidophilus* (ATCC 314), *F. nucleatum* (ATCC 10953), *P. gingivalis* (ATCC 33277) were grown anaerobically (5% CO_2, 100% relative humidity) at 37°C. *Candida albicans* was grown on aerobically on Sabouraud Dextrose Agar media at 37°C.

MIC Determination of Extract

Agar wells (8 mm diameter) were made on each Columbia Blood Agar (containing 5% v/v Sheep Blood) plate and Sabouraud Dextrose Agar media using sterilised cork borer. Columbia Blood Agar was used for growth of all bacteria and Sabouraud Dextrose Agar was used for *Candida albicans*. An aliquot of 100µl of EOTBTC, LU and EOTBTCLU extracts ranging in concentration (6mg/ml to 36mg/ml) were dispensed in agar wells. Different concentrations of extracts were dispensed in each well and diffusion was allowed for 2 hours at 10°C. MIC was determined using Agar well diffusion technique. Homogenous suspension (density 2×10^8 CFU/ml) of bacteria were prepared by mixing log phase culture in sterilized water blank (0.02% Tween 20). The cell density was counted using Neuber's chamber and appropriately diluted. Culture of *Streptococcus mutans* (Sm), *Staphylococcus aureus* (Sa), *Lactobacillus acidophilus* (La), *Fusobacterium nucleatum* (Fn), *Porphyromonas gingivalis* (Pg) and *Candida albicans* (Ca), was uniformly spread on plates such that growth inhibition with each bacteria was tested against all concentration of EOTBTC, LU and EOTBTCLU extract. DMSO and Chlorhexidine (0.2%) were used as negative and positive control, respectively in all experiments. Zone of inhibition (ZoI) formed around the agar wells were measured. Minimum Inhibitory Concentration (MIC) is defined herein as concentration of extract that was able to cause inhibition zone around well. The experiments were performed in triplicate set.

Biofilm Inhibition Assay

Biofilm Formation Results are Presented as the Averages of Three Independent Cultures of 12 Replicates Wells

Anti-biofilm activity of EOTBTCLU extract were studied using commercially available Artificial saliva (AS) and RPMI media supplemented with 5% Fetal Bovine Serum to promote bacterial adhesion. The experiment was carried out in 96 well collagen coated

plates and separate plate was used for different microorganisms. Initially the 200 µL of AS was dispensed in agar well and incubated for a period of 2 hours at 37°C. Thereafter, AS was carefully removed and log phase culture at density 2×10^8 CFU/mL was added to wells. A set 12 wells without addition of extract were marked as control while to other different concentrations of extract was added (replicate of six well per concentration of extract). Incubation of plates were done at 37°C in aerobic or anaerobic conditions (5% CO_2, 100% Relative Humidity) as mentioned above for 72 hours. Thereafter, used broth and unadhered cells were carefully removed from wells followed by washing with 200 µL PBS. The wells were dried for 2–3 hours at room temperature followed by addition of 100 µL Crystal Violet (CV) (0.05% w/v) and incubated for 30 minutes. Destaining was performed using ethanol and Optical density was measured at 600 nm. The results are reported as reduction in absorbance as compared to control (100). The amount of biofilm formed was calculated using below mentioned formula:

$$Amount\ of\ biofilm\ formed = \frac{\text{Average CV optical density corrected for blank} \times 100\ (mm^2)}{\text{Area of base of well}\ (mm^2)}$$

The reduction in Biofilm formation is calculated as:

$$100 - \text{Amount of biofilm formed}\ (\%)$$

The Minimum Reduction in Biofilm (MRB) is defined herein as minimum concentration of extract which caused reduction in biofilm formation by microorganism.

Phytochemical Analysis

Phytochemical composition of ethanolic extracts of EO, TB, TC, LU were studied using standard protocol.[15] The presence of Alkaloids, Anthraquinone, Saponins, Steroids, Phenol, Proteins, Tannins, Sugar/ carbohydrates, Lignans, Linolenic acid, Carotenoid, Unsaturated fatty acids, Cyanogenic glycosides were qualitatively analyzed.

Data Analysis

The results of MIC determination were statistically analysed using SPSS (Statistical Package for Social Sciences) Ver. 20 (IBM, Chicago, USA) and are represented as mean and standard deviation.

Results

Antimicrobial spectrum of EOTBTC, LU and EOTBTCLU extracts against dental caries associated microflora is evident in Fig. 1, Fig. 2 and Fig. 3. The extracts displayed wide range of antimicrobial activity against both aerobic and anaerobic microorganisms tested herein, although the MIC of EOTBTC, LU and EOTBTCLU with different bacteria varied. EOTBTC

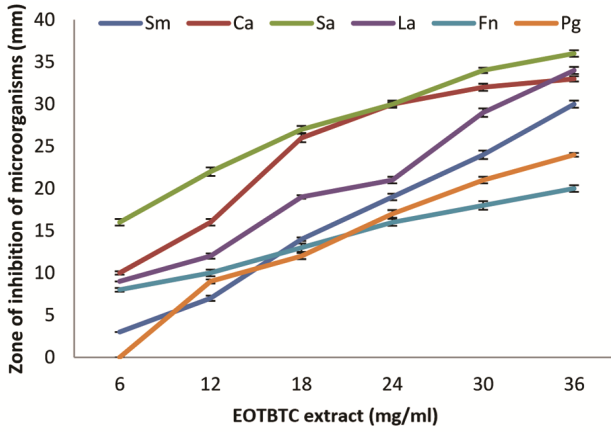

Fig. 1 — Zone of inhibition of microorganism in presence of EOTBTC extract

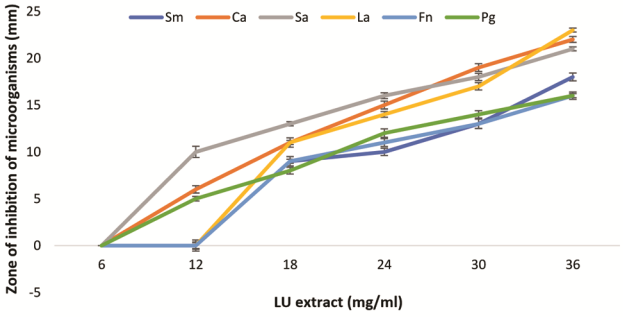

Fig. 2 — Zone of inhibition of microorganism in presence of LU extract

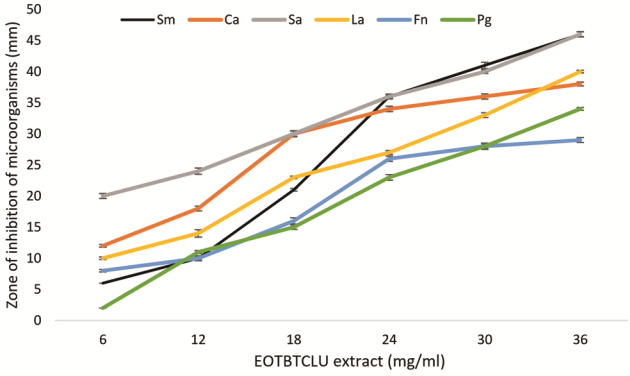

Fig. 3 — Zone of inhibition of microorganism in presence of EOTBTCLU extract

Table 1 — Reduction in Biofilm formation by EOTBTCLU extract

Concentration of EOTBTCLU extract (mg/mL)	Reduction in Biofilm formation (% ± Sd)					
	Streptococcus mutans	*Candida albicans*	*Staphylococcus aureus*	*Lactobacillus acidophilus*	*Fusobacterium nucleatum*	*Porphyromonas gingivalis*
6	0	2.2 ± 1.0	3.8 ± 1.6	0	0	0
12	3.7 ± 2.1	12.54 ± 5.3	13.61 ± 4.2	0	0	0
18	14.8 ± 1.9	22.9 ± 3.3	27.7 ± 4.7	11.5 ± 2.9	18.9 ± 1.53	0
24	39.2 ± 3.2	32.1 ± 0.6	38.33 ± 3.6	26.9 ± 6.2	37.89 ± 2.77	15.6 ± 4.58
30	50.12 ± 2.5	49.7 ± 2.8	51.4 ± 3.18	48.3 ± 2.4	50.9 ± 1.76	22.78 ± 3.97
36	63.21 ± 1.7	58.3 ± 1.9	57.9 ± 4.8	52.45 ± 2.92	53.94 ± 3.28	36.3 ± 4.0
42	65.3 ± 3.9	66.9 ± 1.5	68.4 ± 6.3	55.9 ± 4.12	59.7 ± 1.73	49.98 ± 3.45
48	71.1 ± 8.3	72.6 ± 1.7	76.3 ± 7.6	61.21 ± 3.92	62.5 ± 2.55	52.63 ± 2.67
Chlorhexidine (0.2%)	41.2 ± 4.5	29.7 ± 2.8	33.4 ± 3.27	40.7 ± 2.4	27.9 ± 1.76	26.78 ± 3.17

extract inhibited growth of *Streptococcus mutans, Candida albicans, Staphylococcus aureus, Lactobacillus acidophilus* and *Fusobacterium nucleatum* at 6 mg/mL (MIC) concentration while growth of *Porphyromonas gingivalis* was inhibited at 12 mg/mL (MIC) concentration under *in-vitro* conditions. MIC of 18 mg/mL was noticed in *Streptococcus mutans, Lactobacillus acidophilus* and *Fusobacterium nucleatum* with LU extract while MIC for *Candida albicans, Staphylococcus aureus, Lactobacillus acidophilus* and *Porphyromonas gingivalis* was 12 mg/mL. Zone of inhibition gradually increased with increase in concentration of extracts.

On combining EOTBTC extract with LU extract the MIC with all tested microorganism reduced (Table 2). Also, the cumulative effect of extracts in inhibiting bacteria are remarkable. Chlorhexidine (0.2%) used as negative control, caused zone of inhibition of 17.19 mm (0.09) *Streptococcus mutans*, 17.11mm (0.02), *Candida albicans*, 16.85 mm (0.70) *Staphylococcus aureus*, 17.18 mm (0.07) *Lactobacillus acidophilus*, 16.86 mm (0.61) *Fusobacterium nucleatum*, 17.31 mm (0.02) *Porphyromonas gingivalis*, respectively.

Reduction in biofilm formation by *S. mutans, C. albicans, S. aureus*, *L. acidophilus*, *F. nucleatum*, *P. gingivalis* was noticed with EOTBTCLU extract. Combination of all extracts have shown increase in inhibition of biofilm formation by all caries associated microflora tested herein.

Almost 50% reduction in biofilm of *S. mutans, C. albicans, S. aureus* and *F. nucleatum* was observed at 30 mg/ml concentration of EOTBTCLU extract (Table 1). While with *L. acidophilus* and *P. gingivalis* approximately 50% reduction in biofilm formation occurred at concentration of 36 and 42 mg/mL respectively.

Chemical constituents present in EOTBTC extract and LU extract were qualitatively assessed using standard protocols and the results of same are presented in Table II. It is noteworthy that some of the chemical constituents are of common occurrence in both extract while only few are different.

Table 2 — Phytochemical composition of herbal extracts

Biochemical test	EOTBTC extract	LU extract
Alkaloids	+	+
Anthraquinone	+	+
Saponins	+	-
Steroids	-	-
Phenol	+	+
Protein	W+	+
Tannins	+	+
Sugar/ carbohydrates	-	+
Lignans	+	+
Linolenic acid	+	+
Carotenoid	+	+
Unsaturated fatty acids	-	+
Cyanogenic glycosides	-	+

Discussion

Maintenance of proper oral health is one of the most important factor affecting health of individuals. Some of the common constituents of mouth washes include sodium fluoride (86% of mouth washes), aluminium lactate (1.7% of mouth washes), zinc compounds (19% of mouth washes), Stannous compounds (2% of mouth washes), chlorhexidine (20.7% of mouthwashes), antimicrobial compounds (50–75% of mouth washes), etc., which may cause several side effects and allergies.[16] Continual uninterrupted use of mouthwashes in day to day life have led emergence of drug resistance among microorganisms. Harmful effect of synthetic drugs have re-enforced on importance of Ethnomedicines.[17]

Large scale preparation of herbal extracts with retention of its bioactive constituentc is challenging. Several techniques and solvents have been used to

prepare herbal extracts. It has been reported that cold maceration alcoholic extraction technique when used for extraction of triphala prevented denaturation of heat sensitive phytochemical constituents, and were found to inhibit *C. albicans,* and *Lactobacillus* sp.[8] while Triphala extracts prepared using cold infusion method inhibited growth of *Streptococcus* sp., *Salmonella typhi* and some drug resistant bacteria.[2,9,18] Results of present study supported the previous finding that cold ethanolic extraction technique yield more quantities of bioactive phytoconstituents. Table 2 represents phytoconstituents of EOTBTCLU extract. These constituents include alkaloids, anthraquinone, saponins, phenol, tannins, ascorbic acid, lignans, carotenoid, cyanogenic glycosides, unsaturated fatty acids etc compounds which makes it attractive candidate for controlling growth of variety of microorganisms. Bioactive and antibacterial properties of EOTBTC are attributed by its constituent ingredient comprising of ascorbic acid, astragalin- flavonol, coumarin, chebulic acid, chebulagic acid, emblicol, emblicanin (A, B), ellagic acid, galloyl-glucose, gallic acid-benzenoid, punicalagin, tannin, terchebulin, sitosterol.[18–20] Herbal drugs consisting of mixture of various bioactive compounds exert additive effect wherein these molecules target multiple sites of microorganisms resulting in growth inhibition. The acceptability of herbal sources as drugs is more for long term usage with minimum side effects, they also prevents emergence of drug resistance among microorganism.[21]

The major barrier in treating dental caries is formation of biofilms. These biofilms render drug resistance against various antibiotic. Three herbal drugs namely *Eiekikaryu* S, *Iribakuga* and *Hyakujunro* significantly reduced *Pseudomonas aeruginosa* biofilm formation (P <0.05) under *in vitro* conditions.[22] Sudantha, a herbal extract was also found to restrict formation of biofilms by *Streptococcus mutans* after 4 hours suggesting that inhibition of biofilm formation and its disruption resulted in reduction of biofilm on exposure to herbal extracts.[5] Variety of mechanisms have been suggested for inhibition of biofilm formation on using herbal extracts among which Inhibition of polysaccharide inter-cellular adhesin synthesis due to altered expression of microbial genes is noticeable.[23]

EOTBTCLU extract inhibited growth of not only primary plaque colonisers but also the successive secondary and tertiary plaque colonisers thereby indicating its utility in controlling caries formation process as well as in controlling microbial growth in caries. Reduction in biofilm formation in presence of EOTBTCLU extract suggested that it may find application in controlling and treating dental caries. It may also find application in reducing periodontal problems faced by patients having caries.[24]

Conclusions

The antimicrobial and antibiofilm activity of combination of EOTBTC and LU extract studied herein have highlighted effectiveness of herbal extract for its use in maintaining oral health. Broad spectrum inhibitory effect of EOTBTCLU extract against aerobic, facultative and strict anaerobic microorganisms associated with dental caries makes this study unique in itself. Inhibition in growth of aerobic, facultative and strict anaerobic microorganisms associated with dental plaques by EOTBTCLU extract have emphasized its effectivity in treating deep seated caries and infections. The crude extract used in the study contains many bioactive compound which may provide added advantage as anti-inflammatory and antiallergic effects. Reduction in biofilm by EOTBTCLU extract was found to be remarkable thereby indicating its utility as anti-biofilm agent for controlling and treating dental caries. Development of nanoherbal mouthwash using EOTBTCLU extract will be useful for not only patients with dental problems but also to those have allergies to synthetic chemical mouth washes.

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