Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

Bactericidal activity of ayurvedic formulation against cariogenic microorganisms

Sneha Unnikrishnan, Ramalingam Karthikeyan*

School of Life Sciences, B.S. Abdur Rahman Crescent Institute of Science and Technology, GST Road, Vandalur, Chennai, 600 048, India

ABSTRACT

Dental caries is an infectious disease characterized by formation of biofilm on the teeth. It is a major health concern worldwide and can be prevented. The dried powders of *Acacia arabica*, *Terminalia chebula*, *Terminalia bellerica* and *Emblica officinalis* have the potential to cure oral diseases and these four plant powders have been used in traditional tooth powder formulas in India for more than 100 years. The objective of the present study was to investigate the antimicrobial effect of an aqueous extract of these four plant materials against the cariogenic microorganisms. The Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, kinetics of killing, and adherence assay of the aqueous plant extracts against the cariogenic microorganisms were determined. The results showed that the combined decoction of plant extracts had a high bactericidal activity against all the biofilm forming cariogenic microorganisms tested. Toxicity studies and infection assay on *Bombyx mori* proved that the plant decoctions were not toxic. These assays suggest that the combined decoction could act as a potential mouthwash effective against dental caries.

1. Introduction

Dental caries is a worldwide problem affecting millions of people. It is a microbial infectious disease caused due to the demineralization and remineralization of the tooth surface. The microbes convert sucrose to acids which destroy the tooth enamel by decalcification (Badria and Zidan, 2004). The chief bacteria which cause tooth decay are *Streptococcus mutans*, *Lactobacillus casei* and *Actinomyces viscosus* (Jenkinson and Lamont, 2005).

Mouthwash is a potential inhibitor of dental caries and it containstriclosan, cetylpyridinium chloride, zinc citrate or chlorohexidine. Though they are effective, they have certain side effects like staining of teethand development of antimicrobial strains (Ramalingam and Amaechi, 2018).

A number of plants like orange tree, lime tree, neem plants have been used for years as chewing sticks. Literature has shown that neem has antimicrobial and therapeutic effects suggesting its potential to be used as an endodontic irrigant (Biswas et al., 2002). Murray et al. (2008) also suggested that *Morindacitrifolia* juice can be formulated for use as an intracanal irrigant. Traditional plants like *Acacia arabica* (bark), *Terminalia chebula* (fruits), *Terminalia bellerica* (fruits) and *Emblica officinalis* (fruits) have the potential to treat dental caries and have been used traditionally in India for more than 100 years (Chouhan,

2008). A combination of *T. chebula, T. bellerica* and *E. officinalis,* has a number of potential uses which include free radical scavenging, antioxidant, anti-inflammatory, dental caries prevention, immunomodulating, appetite stimulation, gastric hyperacidity reduction, chemoprotective, antipyretic, antistress, adaptogenic, analgesic, antibacterial, anticandidal, antimutagenic, wound healing, hepatoprotective, anticariogenic, hypoglycemic, anticancer, radioprotective, and chemopreventive effects (Baliga et al., 2012; Shetty et al., 2014). The advantages of using herbal alternatives are cost-effectiveness, easy availability, low toxicity, increased shelf lifeand lack of microbial resistance (Abascal and Yarnell, 2002; Balakrishnan et al., 2018).

The objective of the present study was to investigate the antimicrobial effect of a mouth wash prepared from Triphala in combination with *Acacia arabica*.

2. Materials and methods

2.1. Materials

The dried powders *Acacia arabica* (bark), *Terminalia chebula* (fruits), *Terminalia bellerica* (fruits) and *Emblica officinalis* (fruits)has been used in traditional tooth powder formulation in India for more than 100 years and were purchased from the local traditional stores in Chennai.
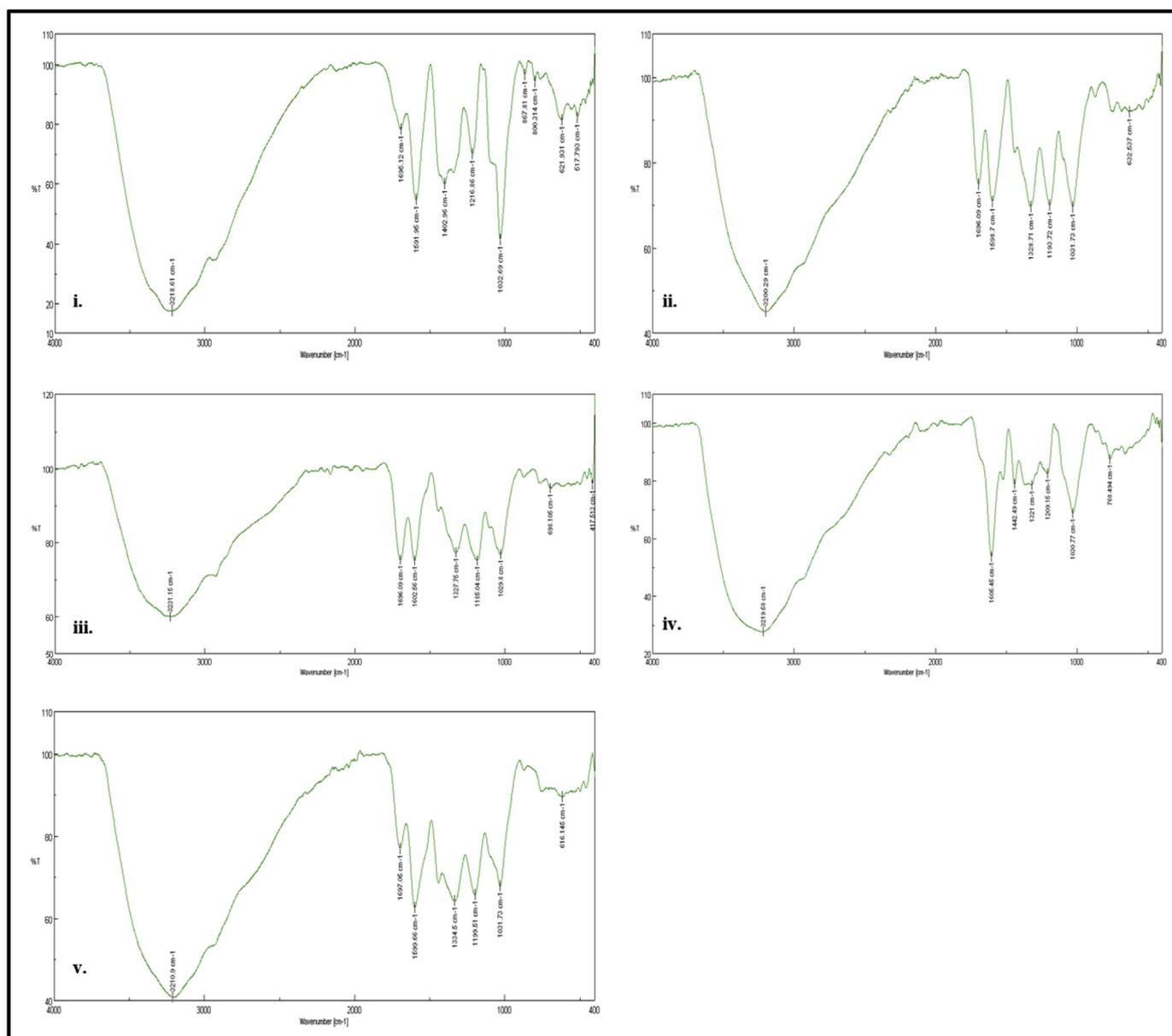

Fig. 1. FTIR spectrum of i. *Emblica officinalis* decoction, ii. *Terminalia chebula* decoction, iii. *Terminalia bellerica* decoction, iv. *Acacia arabica* decoction, v. Mixed decoction.

The powders were of pharmacopoeia standards with reference to "The Ayurvedic Pharmacopoeia of India". These powders were weighed and prepared into individual decoctions as well as in mixed ratio of 7:1:1:1 (*A. arabica* (70%), *T. chebula* (10%), *T. bellerica* (10%), *E. officinalis* (10%)) (Ramalingam and Amaechi, 2018). The three biofilm forming cariogenic bacteria used in this study *Streptococcusmutans* (ATCC 25175),*Lactobacillus casei* (ATCC 393) and *Actinomyces viscosus* (ATCC 15987) were purchased from the American Type Culture Collection (ATCC). The media used for the growth of these bacteria were Brain Heart Infusion (BHI) agar and BHI broth purchased from Himedia.

2.2. Preparation of decoction

Herbal decoction was prepared as per the protocol given in Sarangdhar samhita (Tripathi, 2001). All the individual powders as well as the combined powders were mixed with distilled water and boiled for 5 min on an induction stove till a strong decoction was formed. The overall volume reduced to 1/8th of its original volume. After the decoction was prepared, it was cooled to room temperature. The

decoctions were then filtered and centrifuged at 4000 rpm for 10 min to get a clear solution. The supernatant was stored in falcon tubes for further analysis.

2.3. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR analysis is an important technique used to detect the different bonds present in a compound. The decoctions were dried and the powder was subjected to FTIR analysis in a Jasco FTIR 6300 spectrometerwith a scan range from 400 to 4000 cm^{-1}having a resolution of 4 cm^{-1}to detect different functional groups present in the sample. The different peaks of the various decoction samples were observed and noted.

2.4. Antibacterial activity

The antibacterial activity of all the individual and combined decoctions was assayed against *S. mutans, L. casei* and *A. viscosus* (10^7 CFU/ml) using the agar well diffusion method. The Brain Heart
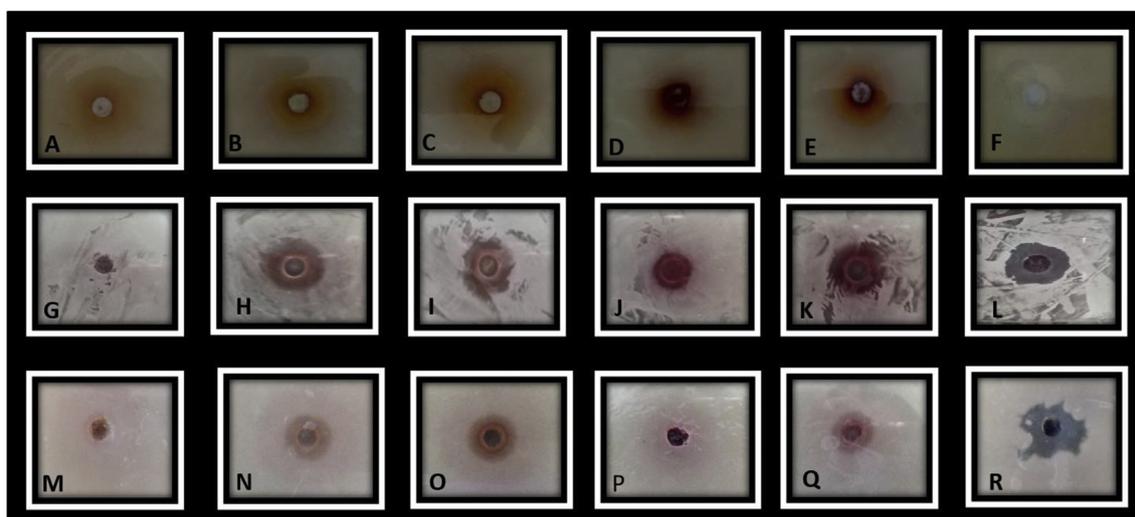
Fig. 2. Photograph of the zone of inhibition of the various microorganisms against the different decoctions. A–F: Zone of inhibition of *Streptococcus mutans* against *Embilica officinalis* Decoction (A), *Terminalia chebula* Decoction (B), *Terminalia bellerica* Decoction (C), *Acacia arabica* Decoction (D), Mixed Decoction (E), Chlorohexidine (F). G–L: Zone of inhibition of *Lactobacillus casei* against *Embilica officinalis* Decoction (G), *Terminalia chebula* Decoction (H), *Terminalia bellerica* Decoction (I), *Acacia arabica* Decoction (J), Mixed Decoction (K), Chlorohexidine (L). M–R: Zone of inhibition of *Actinomyces viscosus* against *Embilica officinalis* Decoction (M), *Terminalia chebula* Decoction (N), *Terminalia bellerica* Decoction (O), *Acacia arabica* Decoction (P), Mixed Decoction (Q), Chlorohexidine (R).
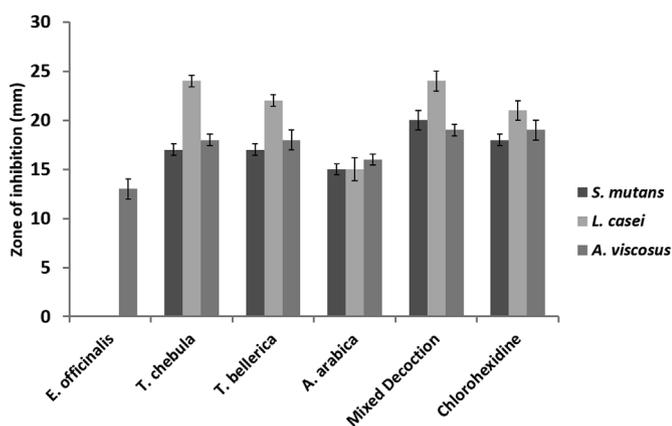

Fig. 3. Graph representing the zone of inhibition of the various microorganisms against the different decoctions.

Infusion (BHI) agar plates were spread with 100 μl of the culture and a well was cut using the cork borer. One hundred microliters of the decoctions were added intothe well and the plates were incubated overnight at 37 °C.The assay was conducted in triplicate.

2.5. MIC and MBC

Plant extract was serially diluted with *S. mutans* cell suspension (10^7 CFU/ml) and was incubated overnight at 37 °C. The lowest concentration showing no growth was recorded as the minimum inhibitory concentration (MIC). For Minimum Bactericidal Concentration (MBC) testing, aliquots were plated onto Brain Heart Infusion (BHI) agar and incubated overnight at 37 °C. The lowest concentration showing 99.9% killing was recorded as the MBC. Chlorohexidine was used as positive control. MIC and MBC assays were conducted for *L. casei* and *A. viscosus*as well. The assays were conducted in triplicate (Karthikeyan et al., 2011; Ramalingam et al., 2012).

2.6. Time kinetics

Overnight cultures of *S. mutans, L. casei, A. viscosus*and mixedcultures of the three strains were added to plant decoctions

inmicrotiter plates. Same concentrations of chlorohexidine were used as positive control. Following addition of the bacterial culture, 100 μl sample was retrieved from each plate at 1, 5, 15, 30 and 60 min and serially diluted (10^{-1}– 10^{-4}). The bacterial count was estimated using plate count method for all the dilutions (Karthikeyan et al., 2011; Ramalingam and Amaechi, 2018). The dilution which showed countable number of microbes was noted along with the count of the bacteria. The experiment was conducted in triplicate.

2.7. Adherence assay

Overnight bacterial cultures containing 10^7 CFU/ml of *S. mutans, L. casei, A. viscosus*and mixed cultures of the three strains were added to BHI broth containing plant decoctions individually and in combination in the ratio 1:1. The tubes were incubated aerobically (to simulate conditions in the mouth) at 37 °C for 24 h at an angle of 30°(Ramalingam et al., 2012; Ramalingam and Amaechi, 2018). Chlorohexidine 0.2% (v/v) was used as positive control. The contents of the tubes were poured out and the tubes were washed with distilled water. One milliliter of autoclaved distilled water was then added to all the tubes and the tubes were vortexed for a minute. Subsequently dilutions were made from this starting from 10^{-1} upto 10^{-4} for all the samples. The bacterial count was estimated at each dilution for all the samples by plate count technique. The dilution which showed countable number of microbes was noted along with the count of the bacteria. All experiments were carried out in triplicate.

2.8. Bombyx mori toxicity infection assay

As an invertebrate model, silkworm model is characterized by its convenience, low cost, no ethical issues. The presence of conserved immune response and similar pharmacokinetics compared to mammals make silkworm infection model suitable to examine the therapeutic effectiveness of antimicrobial agents (Panthee et al., 2017). *Bombyx mori* collected from sericulture units in Alangayam, Tamil Nadu was acclimatized in the lab and fed with mulberry leaves.

For control studies, *Bombyx mori* worms (5 per set) were injected with 200 μL of the plant extract diluted 50% with ultrapure water. Two hundred microliter of chlorohexidine was used as positive control and as a negative control 200 μL of sterile distilled water was injected into
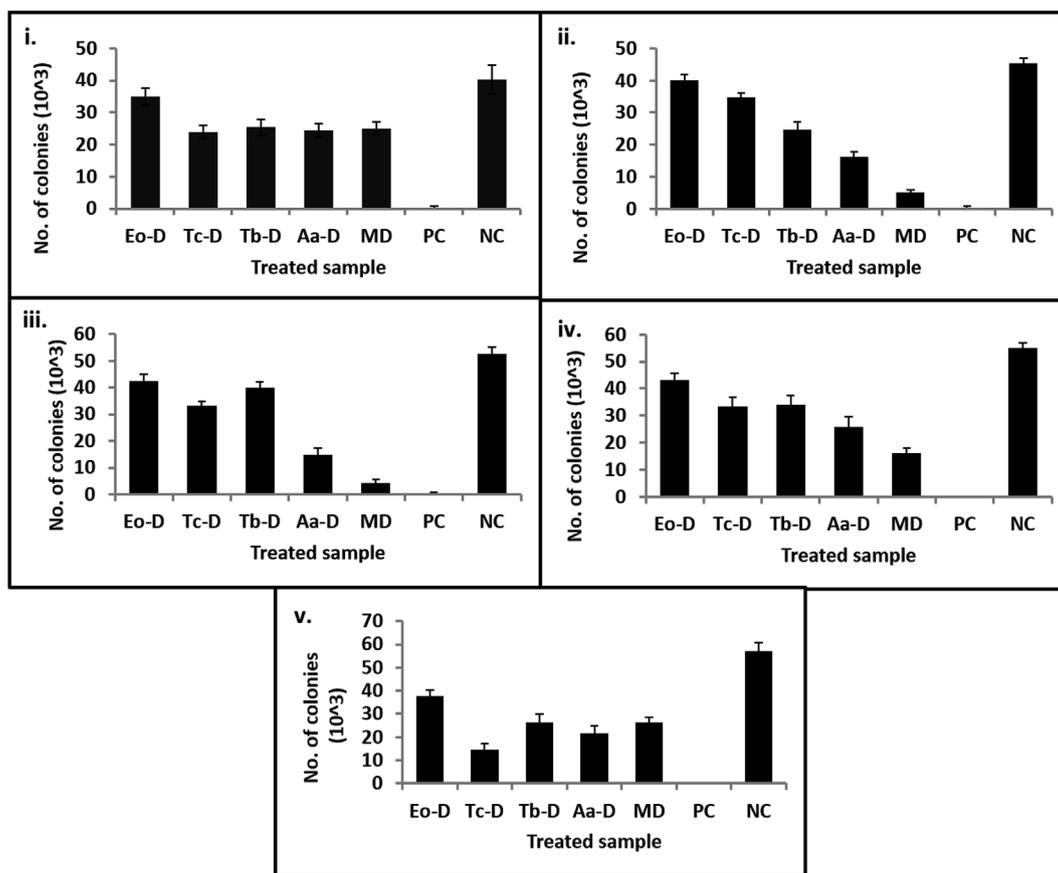
Fig. 4. *Streptococcus mutans* count after treatment with decoctions at i. 1, ii. 5, iii. 15, iv. 30 and v. 60 min. (Eo-D: *Embilica officinalis* Decoction,Tc-D: *Terminalia chebula*Decoction,Tb-D: *Terminalia bellerica*Decoction, *Aa-D: Acacia arabica* Decoction, MD: Mixed Decoction, PC: Positive Control, NC: Negative Control).

the worms. They were incubated for a day at room temperature. The number of worms surviving were noted after 24 h. The experiment was conducted in triplicate (Zhang et al., 2015; Usui et al., 2016).

For carrying out the infection assay, the worms were first injected with 1 unit of bacterial culture *S. mutans*. After 15 min, they were injected with 2 units of the plant decoctions individually or in combination. For the positive control, 2 units of chlorohexidine was injected and for negative control, 2 units of sterile distilled water was injected into the worms. They were incubated for a day at room temperature. The number of worms surviving were noted after 24 h. This assay was repeated with *L. casei, A. viscosus* and combination of all 3 bacteria. The experiment was conducted in triplicate (Zhang et al., 2015; Usui et al., 2016).

All the data was statisticallyanalyzed using one-way ANOVA. Criterion for statistical significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Preparation of decoction

*Acacia arabica, Terminalia chebula, Terminalia bellerica*and *Emblica officinalis* decoctions were prepared individually (Welihinda et al., 2006). A combined decoction of*Acacia arabica, Terminalia chebula, Terminalia bellerica*and *Emblica officinalis* was preparedin the ratio of 7:1:1:1 respectively (Ramalingam and Amaechi, 2018).

3.2. Fourier transform infrared spectroscopy (FTIR) analysis

Fig. 1 represents the various FTIR spectra of the different decoctions. FTIR spectrum of decoction of *Emblica officinalis* showed a major peak at $3218.61 \, cm^{-1}$ which indicates the presence of phenols and

alcohols. The other peaks at $1696.12 \, cm^{-1}$, $1691.96 \, cm^{-1}$, indicates the presence of carboxylic acids, peak at $1402.96 \, cm^{-1}$ indicates alkanes, peaks at $1216.86 \, cm^{-1}$, $1032.69 \, cm^{-1}$ represent esters or ethers, the other peaks at $867.81 \, cm^{-1}$, $800.314 \, cm^{-1}$, $621.931 \, cm^{-1}$, $617.793 \, cm^{-1}$ represent alkyl halides. FTIR spectrum of decoction of *Terminalia chebula* showed a major peak at $3200.29 \, cm^{-1}$ which indicates the presence of phenols and alcohols. The other peaks at $1696.09 \, cm^{-1}$, $1598.7 \, cm^{-1}$, indicates the presence of carboxylic acids, peak at $1328.71 \, cm^{-1}$ indicates alkanes, peaks at $1193.72 \, cm^{-1}$, $1031.73 \, cm^{-1}$ and $632.637 \, cm^{-1}$ represent alkyl halides. FTIR spectrum of decoction of *Terminalia bellirica*showed a major peak at $3231.16 \, cm^{-1}$ which indicates the presence of phenols and alcohols. The other peaks at $1696.09 \, cm^{-1}$, $1602.66 \, cm^{-1}$ indicate the presence of carboxylic acids, peak at $1327.76 \, cm^{-1}$ indicates alkanes, peaks at $1186.04 \, cm^{-1}$, $1029.8 \, cm^{-1}$ and $417.513 \, cm^{-1}$ represent alkyl halides, and peak at $698.105 \, cm^{-1}$ represents alkynes. FTIR spectrum of decoction of *Acacia arabica* showed a major peak at $3219.68 \, cm^{-1}$ which indicates the presence of phenols and alcohols. The other peaks at $1606.45 \, cm^{-1}$, $1442.49 \, cm^{-1}$ indicate the presence of carboxylic acids, peaks at $1321 \, cm^{-1}$, $1209.16 \, cm^{-1}$, $1030.77 \, cm^{-1}$ and $768.494 \, cm^{-1}$ represent alkyl halides. FTIR spectrum of decoction of mixed decoction of all 4 plants showed a major peak at $3210.9 \, cm^{-1}$ which indicates the presence of phenols and alcohols. The peak at $1697.05 \, cm^{-1}$ indicates the presence of aldehydes, peak at $1599.66 \, cm^{-1}$ represents amides, peaks at $1334.6 \, cm^{-1}$, $1199.61 \, cm^{-1}$, $1031.73 \, cm^{-1}$ and $616.145 \, cm^{-1}$ represent alkyl halides.

FTIR analysis of the Triphala showed the presence of different functional groups ranging from O−H alcohol (3200-$3600 \, cm^{-1}$), C−H alkane (2850-$3000 \, cm^{-1}$), =C−H aldehyde (2820-$2850 \, cm^{-1}$), acyclic ketone (1705-$1725 \, cm^{-1}$), N−H amide (1550–1640 bending), C=C aromatic (1400-$1600 \, cm^{-1}$), C−N amine (1080-$1360 \, cm^{-1}$), C−F alkyl
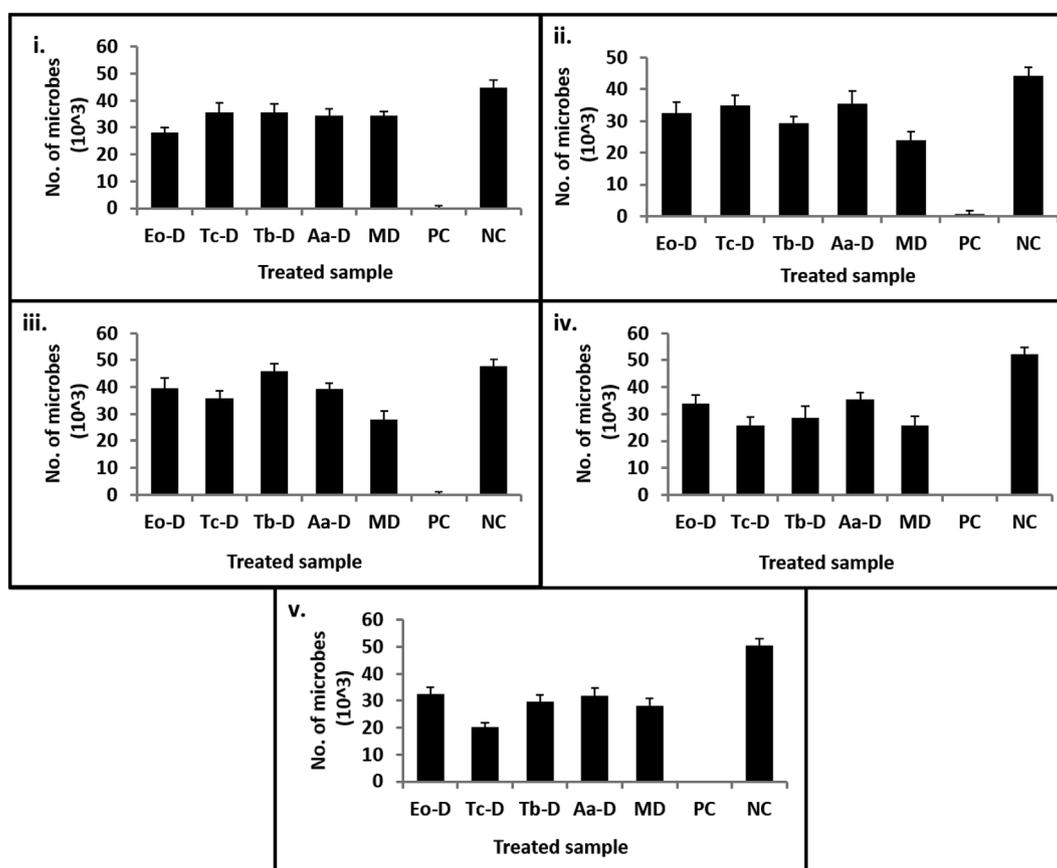
Fig. 5. *Lactobacillus casei* count after treatment with decoctions at i. 1, ii. 5, iii. 15, iv. 30 and v. 60 min.

Halide (1000-1400 cm^{-1}), =C−H alkene (675-1000 cm^{-1}) as functional groups (Amala and Jeyaraj, 2014).

3.3. Antibacterial activity

The antimicrobial activity of plant decoctionswas checked against *Streptococcus mutans, Lactobacillus casei*and *Actinomyces viscosus*. It wasquantitatively assessed by measuring the inhibition zone. Plant extract was effective with the inhibition zones ranging from 13 to 24 mm. The mixed decoction had a maximal zone of inhibition of 20 mm, 24 mm and 19 mm when compared to chlorohexidine which showed a zone of inhibition of 18 mm, 21 mm and 19 mm against *Streptococcus mutans, Lactobacillus casei*and *Actinomyces viscosus*respectively (p < 0.05). Hence, combined decoction is a better antibacterial agent in comparison to chlorohexidine. Figs. 2 and 3 represent the photograph and graph of the zone of inhibition of the different microbes against the different decoctions.

Invitro antimicrobial activity of Triphala (aqueous extract)with *Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* has been reported to have inhibitory zone at 14 mm, 10.75 mm, 10 mm (Amala and Jeyaraj, 2014). Gupta et al. (2014) has reported that the combined aqueous extract of *Terminalia chebula, Emblica officinalis* and *Terminalia bellerica* showed higher zone of inhibition compared to 0.2% chlorohexidine. Thomas et al. (2011) found the mean inhibitionzone for the aqueous extract of Triphala (combination of *Emblica officinalis, Terminalia chebula* and *Terminalia bellerica*)at 50%, 25% and 12.5% against *S. mutans* (MTCC strain) to be 30 mm, 28 mm, and 24 mm respectively. The mean inhibition zone for the aqueousextract of Triphala at 50%, 25% and 12.5% against clinicalisolates of *S. mutans* was found to be 34 mm, 30 mm, and 28 mm, respectively. Prajapati and Raol (2014) found the aqueous extract of Triphala to inhibit *S. mutans*. The mean inhibition zone was 17 mm against MTCC strains and 19 mm

against clinicalisolates.

3.4. MIC and MBC

The MIC assay was performed for all the three bacteria. All the three bacteria showed inhibition upto the 3rd well (p < 0.05). So upto 3 fold dilution there was inhibition. Hence the MIC was at 1.25 mg/ml.

MBC was performed from 1 to 6 wells for all the samples along with negative control and media control. The concentration up to which there was 99.9% reduction in bacterial load was considered to be as MBC. The concentration of MBC was 2.5 mg/ml (p < 0.05).

Triphala showed minimal inhibitory concentration and minimal bactericidal concentration at 6.25% for MTCC strain of *S. mutans* and 3.12% for clinical isolate of *S. mutans* (Thomas et al., 2011). MICs and MBCs of the colloidal solutions containing ZnO, CuO, TiO$_2$ and Ag nanoparticles were comparable to each other and all were significantly lower than that of the chlorhexidine mouthrinse against both *S. mutans* and *S. sangius* (Ahrari et al., 2015).

3.5. Kinetics study

Kinetics study at time points 1, 5, 15, 30 and 60 min was performed for all the bacteria-individually and in combination. Throughout the time period, the number of colonies in the decoction treated samples was much lesser when compared to the negative control (p < 0.05). The number of bacterial colonies increased rapidly from 1 min to 60 min in the negative control. In all the treated samples, the number of microbes was gradually increased from 1 min to 15 min followed by gradual reduction (p < 0.05). The positive control did not contain any bacterial colonies in all the cases. Figs. 4–7 represent the graphs of the *Streptococcus mutans, Lactobacillus casei, Actinomyces viscosus* and combination of all three bacterial cultures after treatment with various
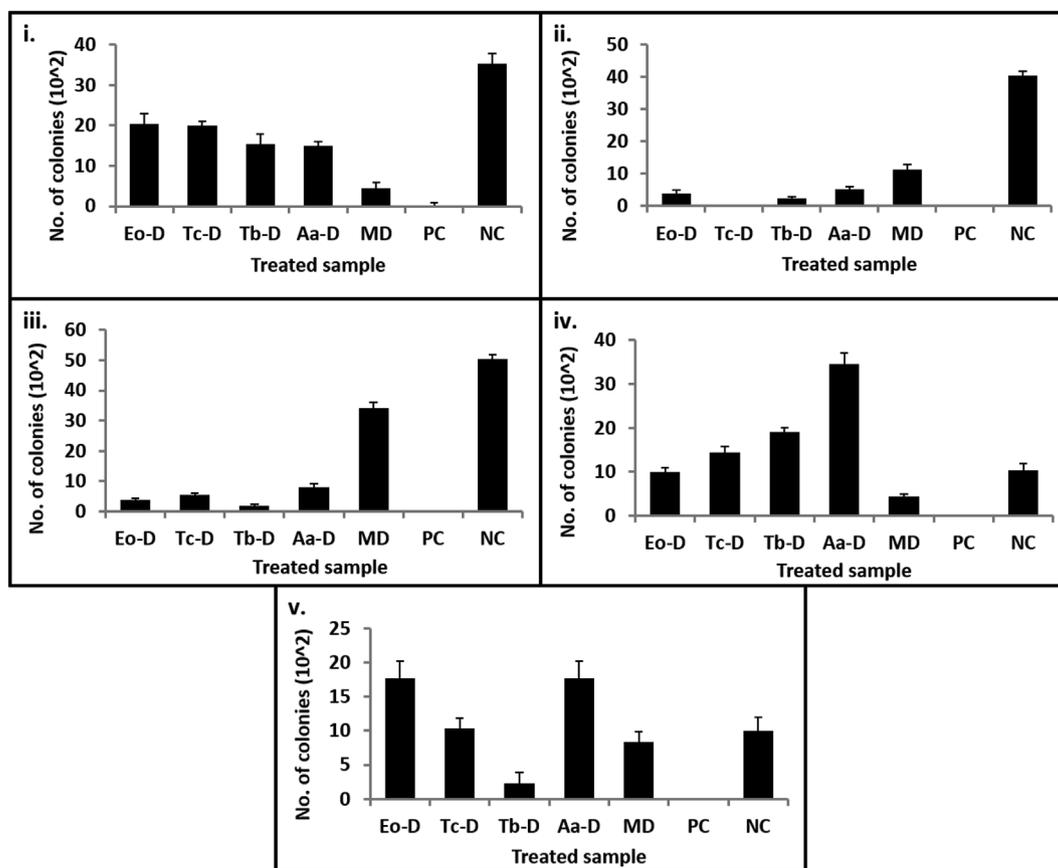
Fig. 6. *Actinomyces viscosus* count after treatment with various decoctions at i. 1, ii. 5, iii. 15, iv. 30 and v. 60 min.

decoctions. The combined decoction Nanoemulsions have been found to have bactericidal properties against Grampositive and enteric pathogen species in various earlier reports (Teixeira et al., 2007; Hamouda et al., 1999, 2001). Greater killing (49–94.73%) of *S. mutans* was observed with mixed herbal powder extract containing methanolic extract of triphala and *Acacia arabica* at 60 min when compared with chlorohexidine (36.56–65.93%) during kinetics study (Ramalingam and Amaechi, 2018).

3.6. Adherence assay

Adherence assay was performed with all the 3 bacteria both individually and as a consortium- *Streptococcus mutans*, *Lactobacillus casei* and *Actinomyces viscosus*. The number of bacterial colonies of all dilutions was noted the next day. In all the 4 cases, the number of bacterial colonies when treated with the various decoctions showed much lesser bacterial count when compared to the negative control which was untreated (p < 0.05). The number of bacterial colonies after being treated with the mixed decoction was 1000-fold lesser than the negative control bacterial count in all the cases. The positive control showed absence of bacteria in all the cases (p < 0.05). Fig. 8 represents the graphs of bacterial count of various treated samples.

Glass adherence assay used in the present study is an effective model for assessing sucrose dependent bacterial adhesion (Nostro et al., 2004). More inhibition of *S. mutans* adherence by mixed herbal powder extract containing methanolic extract of triphala and *Acacia Arabica* (up to 85.64%) at 100 µg/ml concentration has been reported when compared tochlorohexidine which showed inhibition up to 73.21% (Ramalingam and Amaechi, 2018).

3.7. Bombyx mori infection assay

This study is the first study in which plant decoctions have been tested against cariogenic microbes *in vivo* using silk worm as a model organism. In control studies, the photographs of the worms after 24 h of treatment are shown in Fig. 9. Fig. 10 represents the graph indicating the percentage of surviving population of *B. mori* worms after 24 h of administration of the 50% diluted decoctions and chlorohexidine.

In the control, the worms given only plant decoctions survived 100% (p < 0.05). The worms injected with distilled water also survived 100%. The worms injected with chlorohexidine survived only 60% (p < 0.05). This proved that the plant decoctions were not toxic whereas the chlorohexidine was toxic. Side effects of chlorohexidine like altered taste perception, metallic taste, and staining of teeth have been reported earlier (Flötra et al., 1971). Chlorohexidine shows cytotoxicity and even causes dysplasia of the oral mucosa (Sonis et al., 1978; Cabral and Fernandes, 2007; De Souza et al., 2007).

In *S. mutans* infection assay, worms treated with *Terminalia bellerica* and mixed decoction showed higher survival rates (65%) compared to untreated control as well as worms treated with chlorohexidine-both of which showed only 45% survival (p < 0.05). This showed that treatment with mixed decoction was better than chlorohexidine. In all the other bacterial (*L. casei*, *A. viscosus* and mixed culture of all 3 bacteria) infections, the plant decoctions showed better survival rate than the positive control (p < 0.05). Saxena et al. (2017) had reported that the mean colony forming units of *S. mutans* with Triphala was significantly reduced at 5 min when tested on human trials. The ability of these rinses to reduce plaque, freshen breath, to prevent or control tooth decay, to reduce gingivitis, to reduce the speed that tartar forms on the teeth, or to produce a combination of these effects (Kornman, 1986).
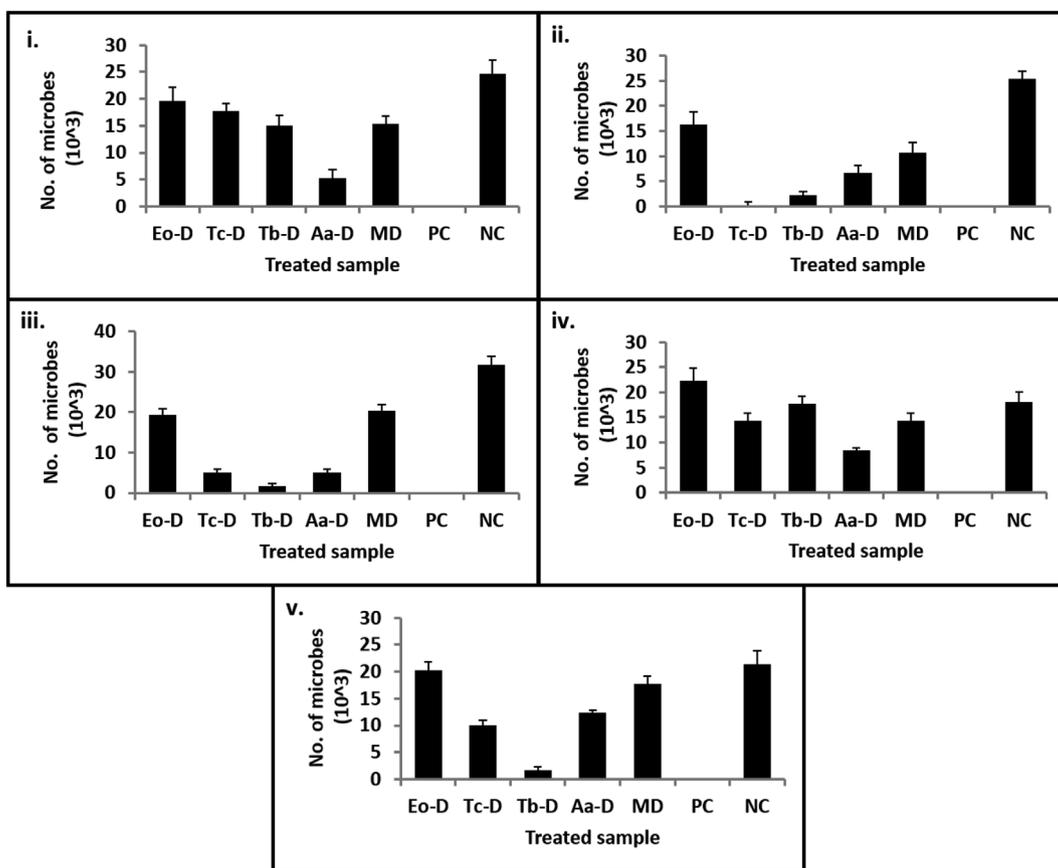
Fig. 7. Combined microbial count after treatment with decoctions at i. 1, ii. 5, iii. 15, iv. 30 and v. 60 min.
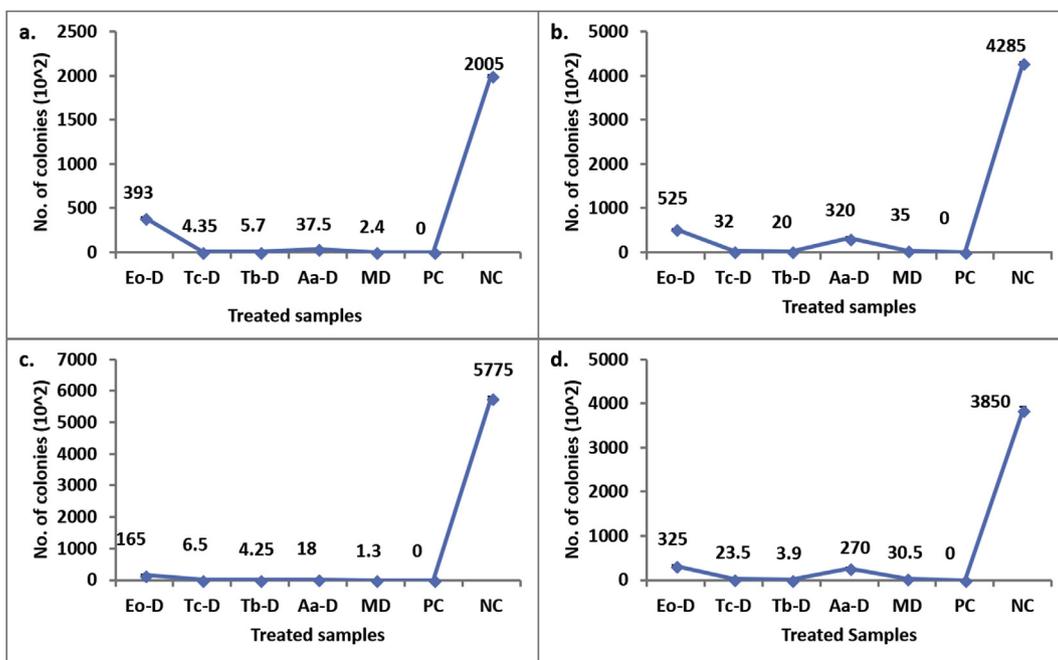

Fig. 8. Microbial count after the adherence assay using drop plate method in a. *Streptococcus mutans*, b. *Lactobacillus casei*, c. *Actinomyces viscosus* and d. combined bacteria. (Eo-D- *Embilica officinalis* Decoction,Tc-D-*Terminalia chebula*Decoction,Tb-D-*Terminalia bellirica* Decoction, Aa-D- *Acacia arabica* Decoction, MD- Mixed Decoction, PC− Positive Control, NC− Negative Control).

4. Conclusion

Thus the aqueous plant extract prepared from the plants- *Acacia arabica*, *Terminalia chebula*, *Terminalia bellerica* and *Emblica officinalis*

was found to be effective against the common dental caries causing bacteria- *Streptococcus mutans*, *Lactobacillus casei* and *Actinomyces viscosus*. In this study, antibacterial activity, MIC, MBC, kinetics study, adherence assay and toxicity studies was performed. Commercially

Fig. 9. Photograph of *Bombyx mori* after 24 h of treatment. A, B, C— Control toxicity study with mixed decoction, chlorohexidine and distilled water; D, E, F— *S. mutans*; G, H, I— *L. casei*; J, K, L- *A. viscosus*; M, N, O— combined microbe infection assay using mixed decoction, chlorohexidine and distilled water respectively.
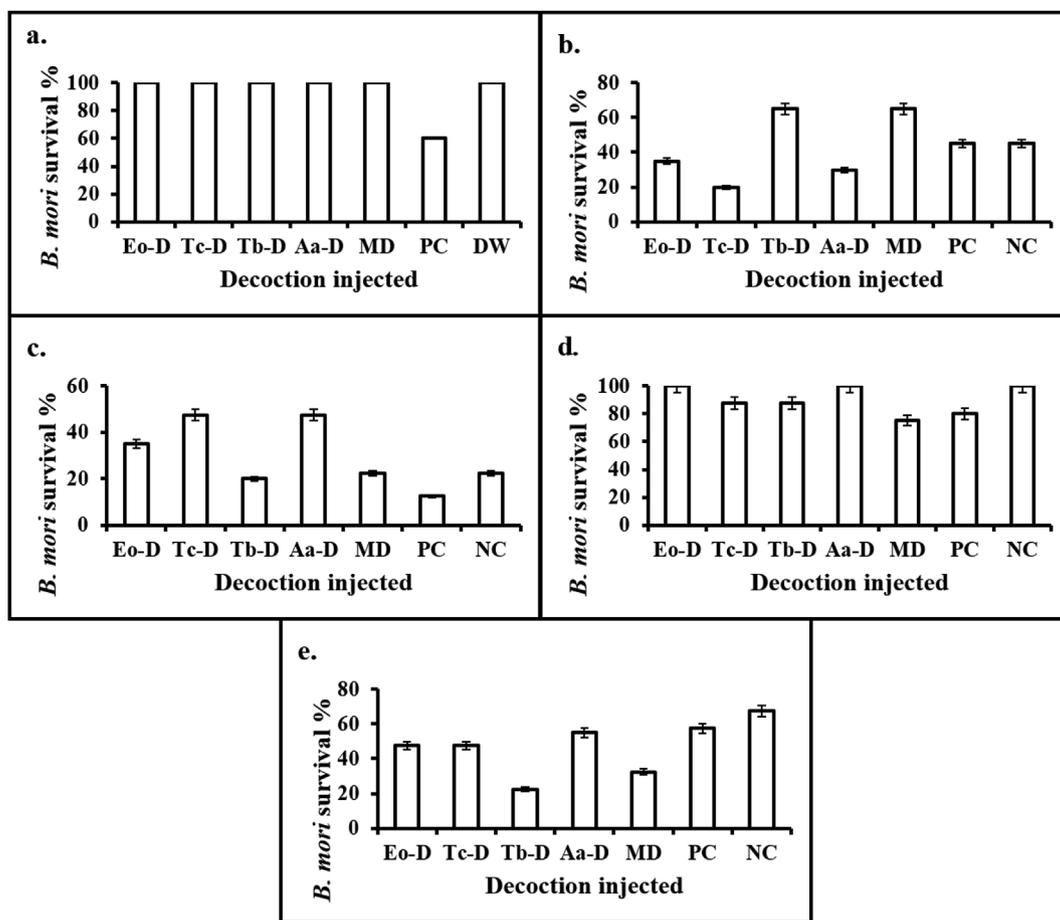

Fig. 10. Control graph represents the surviving population (%) of *B. mori* worms after 24 h of administration of the 50% diluted decoctions and chlorohexidine (control toxicity study),b. *Streptococcus mutans,* c. *Lactobacillus casei,* d. *Actinomyces viscosus*and e. combination of all three micro-organisms infection assay with *Bombyx mori.* (Eo-D: *Embilica officinalis* Decoction,Tc-D: *Terminalia chebula*Decoction,Tb-D: *Terminalia bellerica*Decoction, Aa-D: *Acacia arabica* Decoction, MD: Mixed Decoction, PC: Positive Control, NC: Negative Control).

available chlorohexidine seemed to be an effective antibacterial agent but wascytotoxicand led to reduced survival of *B. mori*. The combined plant decoction gave 99.9% reduction in the total amount of bacteria while proving to be completely non-toxic to *B. mori*.

Funding

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101026.

References

Abascal, K., Yarnell, E., 2002. Herbs and drug resistance Part 2 - clinical implications of research on microbial resistance to antibiotics. Altern. Compl. Ther. 8, 284–290.

Ahrari, F., Eslami, N., Rajabi, O., Ghazvini, K., Barati, S., 2015. The antimicrobial sensitivity of *Streptococcus mutans* and *Streptococcus sangius* to colloidal solutions of different nanoparticles applied as mouthwashes. Dent. Res. J. 12 (1), 44–49.

Amala, V.E., Jeyaraj, M., 2014. Determination of antibacterial, antifungal, bioactive constituents of Triphalaby FT-IR and GC-MS analysis. Int. J. Pharm. Pharmaceut. Sci. 6 (8), 123–126.

Badria, F.A., Zidan, O.A., 2004. Natural products for dental caries prevention. J. Med. Food 7, 381–384.

Balakrishnan, B., Nair, R., Perayil, J., Fenol, A., Menon, L., Vyloppillil, R., Bhaskar, A., Shereef, M., George, M., Suresh, R., 2018. Triphala ayurvedic mouth rinse – rediscovering the past. J. Ayurveda Integr. Med. 9 (2) sup. 1.

Baliga, M.S., Meera, S., Mathai, B., Rai, M.P., Pawar, V., Palatty, P.L., 2012. Scientific validation of the ethnomedicinal properties of the Ayurvedic drug Triphala: a review. Chin. J. Integr. Med. 18 (12), 946–954.

Biswas, K., Chattopadhyay, I., Banerjee, R.K., Bandyopadhyay, U., 2002. Biological activities and medicinal properties of neem (*Azadirachtaindica*). Curr. Sci. 82, 1336–1345.

Cabral, C.T., Fernandes, M.H., 2007. In vitro comparison of chlorhexidine and povidone–iodine on the long-term proliferation and functional activity of human alveolar bone cells. Clin. Oral Invest. 11, 55–164.

Chouhan, B., 2008. Triphala: a comprehensive ayurvedic review. Int. J. Res. Ayurveda Pharm. 4 (4), 612–617.

De Souza, L.B., de Aquino, S.G., de Souza, P.P., Hebling, J., Costa, C.A., 2007. Cytotoxic effects of different concentrations of chlorhexidine. Am. J. Dent. 20 (6), 400–404.

Flötra, L., Gjermo, P., Rölla, G., Waerhaug, J., 1971. Side effects of chlorhexidine mouth washes. Eur. J. Oral Sci. 79, 119–125.

Gupta, R., Chandrashekar, B.R., Goel, P., Saxena, V., Hongal, S., Jain, M., Ganavadiya, R., 2014. Antimicrobial efficacy of aqueous and ethanolic extracts of Triphala on primary plaque colonizers: an *in vitro* study. J. Young Pharm. 6 (3), 7–13.

Hamouda, T., Hayes, M.M., Cao, Z., 1999. A novel surfactant nanoemulsion with broad-spectrum sporicidal activity against *Bacillus* spores. J. Infect. Dis. 180, 1939–1949.

Hamouda, T., Myc, A., Donovan, B., Shih, A.Y., Reuter, J.D., Baker, J.R., 2001. A novel surfactant nanoemulsion with a unique non-irritant topical antimicrobial activity against bacteria enveloped viruses and fungi. Microbiol. Res. 156, 1–7.

Jenkinson, H., Lamont, R.J., 2005. Oral microbial communities in sickness and in health. Trends Microbiol. 13, 589–595.

Karthikeyan, R., Amaechi, B.T., Rawls, H.R., Lee, V.A., 2011. Antimicrobial activity of nanoemulsion on cariogenic *Streptococcus mutans*. Arch. Oral Biol. 56, 437–445.

Kornman, K.S., 1986. The role of supragingival plaque in the prevention and treatment of periodontal diseases. A review of current concepts. J. Periodontal. Res. 21 (Suppl. l), 522.

Murray, P.E., Farber, R.M., Namerow, K.N., Kuttler, S., Garcia-Godoy, F., 2008. Evaluation of *Morindacitrifolia* as an endodontic irrigant. J. Endod. 34, 66–70.

Nostro, A., Cannatelli, M.A., Crisafi, A.D., Musolino, A.D., Procopio, F., Alonzo, V., 2004. Modifications of hydrophobicity, *in vitro* adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysumitalicum* extract. Lett. Appl. Microbiol. 38, 423–427.

Panthee, S., Paudel, A., Hamamoto, H., Sekimizu, K., 2017. Advantages of the silkworm as an animal model for developing novel antimicrobial agents. Front. Microbiol. 8, 373.

Prajapati, R.A., Raol, B.V., 2014. The study on the efficacy of some herbal extracts for the control of dental caries pathogen-*Streptococcus mutans*. Int. J. Pharmaceut. Sci. Health Care 1, 49.

Ramalingam, K., Amaechi, B.T., 2018. Antimicrobial effect of herbal extract of Acacia Arabica with Triphala on the biofilm forming cariogenic microorganisms. J. Ayurveda Integr. Med. https://doi.org/10.1016/j.jaim.2018.01.005.

Ramalingam, K., Amaechi, B.T., Rawls, H.R., Lee, V.A., 2012. Antimicrobial activity of nanoemulsion on cariogenic planktonic and biofilm organisms. Arch. Oral Biol. 57, 15–22.

Saxena, S., Lakshminarayan, N., Gudli, S., Kumar, M., 2017. Anti-bacterial efficacy of *Terminalia chebula, Terminalia bellirica, Embilica officinalis* and triphala on salivary *Streptococcus mutans* count – a linear randomized cross over trial. J. Clin. Diagn. Res. 11 (2), ZC47–ZC51.

Shetty, P.J., Hegde, V., Gomes, L., 2014. Anticandidal efficacy of denture cleansing tablet, *Triphala, Aloe vera*, and Cashew leaf on complete dentures of institutionalized elderly. J. Ayurveda Integr. Med. 5, 11–14.

Sonis, S.T., Clark, W.B., Shklar, G., 1978. Chlorhexidine-induced lingual keratosis and dysplasia in rats. J. Periodontol. 49 (11), 585–591.

Teixeira, P.C., Leite, G.M., Domingues, R.J., Paul, J.S., Gibbs, A., Ferreira, J.P., 2007. Antimicrobial effects of a microemulsion and a nanoemulsion on enteric and other pathogens and biofilms. Int. J. Food Microbiol. 118, 15–19.

The Ayurvedic Pharmacopoeia of India, 2016. first ed., published by Pharmacopoeia Commission of Indian Medicine and Homeopathy.

Tripathi, B., 2001. Sharangdharsamhita, second edition. Chaukhambaprakashan madhyamkhand 2/107.

Thomas, B., Shetty, S., Vasudeva, A., Shetty, V., 2011. Comparative evaluation of Antimicrobial Activity of Triphala and commercially available toothpastes: an *in-vitro* study. Int. J. Public Health Dent. 2, 8–12.

Usui, K., Nishida, S., Sugita, T., Ueki, T., Matsumoto, Y., Okumura, H., Sekimizu, K., 2016. Acute oral toxicity test of chemical compounds in silkworms. Drug Discov. Ther. 10, 57–61.

Welihinda, J., Ariyawansa, H.A., Wickramasinghe, R., 2006. Comparison of the diuretic effects of frusemide and the KaraviPanchaka Ayurveda decoction. Ceylon Med. J. 51 (3), 93–97.

Zhang, X.L., Xue, R.Y., Cao, G.L., Pan, Z.H., Zheng, X.J., Gong, C.L., 2015. Silkworms can be used as an animal model to screen and evaluate gouty therapeutic drugs. J. Insect Sci. 12, 4.