WILEY'S

International Journal of Dentistry and Oral Health

ISSN 2471-657X

Research Article Open Access

Estimation of Antibacterial Activity of Plants Extracts from Phyllanthus Emblica, Terminalia Chebula and Eucalyptus Globulus Against Oral Pathogens

Asimuddin M, Bhumika Ranawat, Kaiser Jamil* & Chandrakanth Rao

Head of Dental Department, Bhagwan Mahavir Hospital & Research Centre, Hyderabad, India

Corresponding author: Kaiser Jamil, Head of Dental Department, Bhagwan Mahavir Hospital & Research Centre, Hyderabad, India. Tel: +91-9676872626, E-mail: kj.bmmrc@gmail.com

Citation: Kaiser Jamil et al. (2017), Estimation of Antibacterial Activity of Plants Extracts from Phyllanthus Emblica, Terminalia Chebula and Eucalyptus Globulus Against Oral Pathogens. Int J Dent & Oral Heal. 3:9, 100-104.
DOI: 10.25141/2471-657X-2017-9.0087

Received: October 05, 2017; **Accepted:** October 16, 2017; **Published:** November 30, 2017

Abstract

Dental caries and periodontal disease are the most common oral infections in humans. These diseases are primarily treated with antimicrobial drugs; however, the rise in antimicrobial resistance necessitates the investigation of plant-based phytocompounds as antimicrobial agents. This study investigated the phytochemicals present in three plants, Phyllanthus emblica, Terminalia chebula and Eucalyptus globulus, as well as their antibacterial effect on the cariogenic bacteria, Staphylococcus aureus (MTCC 3160), Escherichia coli (MTCC 1655) and Streptococcus mutans (MTCC 890). Fruits of Phylanthus emblica, Terminalia chebula and leaves of Eucalyptus globulus were extracted in aqueous, ethanol and methanol solvents. These extracts were tested for phytochemical compounds. Antibacterial activity was evaluated using agar disc diffusion method. The minimum inhibitory concentration (MIC) was measured via 96-well turbidimetric method. Results of preliminary qualitative phytochemical study of extracts revealed the presence of tannins and terpinoids in all the three plants. Antibacterial activity assay showed that the plant extracts were more active against gram-positive bacteria than gram-negative bacteria. The most susceptible bacteria was S. aureus. The bacterial strains tested were more susceptible to methanol extracts followed by ethanol and the least effective was aqueous extract. These findings indicate that Phyllanthus emblica, Terminalia chebula and Eucalyptus globulus extracts have potential antibacterial activity against oral pathogens such as S. aureus, S. mutans and E.coli and could be potentially used treatment of dental carries.

Key Words : Denal Caries, Phyllanthus Emblica, Terminalia Chebula And Eucalyptus Globulus, Antimicrobial Activity, Cariogenic Bacteria

Introduction

The normal microflora of the human mouth consists of a complex microbial community of a few eukaryotic fungi and protists, with bacteria as the major microbial component. The most common oral bacteria include streptococci, lactobacilli, staphylococci, corynebacteria and various anaerobes. Streptococci and gram-positive rods comprise the majority of the total viable count (Hamada and Slade, 1980; Aas et al., 2005). Most individuals suffer at some time in their life from localized episodes of disease in the mouth caused by the imbalance in the composition of their natural oral flora (Marsh et al., 2009). These diseases include dental caries, periodontal diseases and oral candidiasis with untreated dental caries commonly affecting humans worldwide (Frencken et al., 2017).

Dental caries erode and destroy the hard dental tissue and usually start in the enamel as a chalky white spot gradually spreading in depth. It affects dentin and in latter stages leads to inflammation of the dental pulp (pulpitis). Dental caries is most prevalent in Asian and Latin American countries and least prevalent in Africa. In India, dental caries affect nearly 60–70% of children (Ingle et al., 2014). Although dental caries is a complex chronic disease with a wide range of biological, environmental and behavioural determinants that complicate its etiology, its pathogenesis is relatively well understood.

Dental caries and periodontal diseases are considered as a serious public health problem and impose a large burden to health care services around the world in mainly in developing countries (Owlia,

et al., 2009). Control and prevention of bacteria, predominantly Streptococcus mutans, Staphylococcus aureus, Escherichia coli, found in biofilms (dental plaque) (Tahir et al., 2012), is crucial for control of dental caries. This can be achieved by controlling plaque formation or removing fully formed plaque. The preventative measures commonly used are chemical agents, which have antimicrobial properties (Nisengard and Newman, 1994). On the other hand, devel¬opment of resistance against antibiotics and antiseptics is a growing cause of concern, limiting their use (Fair and Tor, 2014). In addition, antibiotics are sometimes associated with adverse side effects on the host, which include hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reaction. Therefore, there is a need for new antimicrobial agents.

Plant based antimicrobials represent a vast untapped source of medicine. Plant-based antimicrobials have enormous therapeutic potential as they can serve as the purpose without any side effects that are often associated with synthetic antimicrobials (Hussain and Gorsi, 2004; Ivanova et al., 2005). Extraction and characterization of several active phytocompounds from plants has led to some high activity profile drugs (Singh et al., 2016). Phytochemical screening of plants has revealed the presence of numerous chemicals including alkaloids, tannins, flavanoids, steroids, saponins etc. Many plant extracts and phytochemicals show antioxidants/free radical scavenging properties (Nair et al., 2007; Parekh and Chanda, 2007; Iqbal et al., 2015). Secondary metabolites of plants serve as defense mechanism against predation by many microorganisms, insects and herbivores (Marjorie, 1999; War et al., 2012). Our earlier study on antibacterial activity of zinc oxide nanoparticles synthesized via biological method using Azadirachta indica extract showed potent antibacterial activity against Escherichia coli and Streptococcus mutans (Issa et al., 2015).

Studies have investigated the effect of plant extracts and plant products against oral pathogens and the ability of the plant to inhibit the adhesion of pathogenic microorganisms to the surface of the tooth thereby inhibiting biofilm formation (Palombo, 2009).

Phyllanthus emblica is a deciduous tree found in the subtropical and tropical regions of Asian countries. Studies on the plant extracts have reported potent antimicrobial, antioxidant, anticancer and anti-inflammatory activity (Kumar et al., 2016). Terminalia chebula is extensively used in ayurveda, unani and homoeopathic system. The fruit extract have been reported to possess antiviral, antibacterial, and anticancer and antioxidant activity (Aquil et al., 2005). Eucalyptus species are well known as medicinal plants because of their biological and pharmacological properties. The most important and reported species is Eucalyptus globulus, a major source of essential oils. These oils are used as anesthetics, disinfectants, febrifuges, deodorants, vermifuges, abscesses, asthma (Bajaj et al., 1995). Some antiseptic mouthwashes use eucalyptus, along with other oils, and have been shown to prevent plaque and gingivitis. Other uses include treatment of wounds, burns, ulcers, and cancer (Bachir et al., 2012).

In the present study, we performed phytochemical analysis and investigated the antimicrobial activities of aqueous, ethanol and methanol extracts of these three plants against three major cariogenic microbes, S. mutans, S. aureus, E. coli.

Materials and Methods

Plant material and Plant collection

The fruits of Phyllanthus embilica and Terminalia chebula were purchased from authenticated botanists. The fruits were allowed to dry, the seeds were separated and the remaining part was made into fine powder. After air-drying at room temperature, the leaves of Eucalyptus globulus were crushed into a fine powder.

Preparation of extracts

Aqueous extraction

All the dried plants were materials weighed ten grams and extracted in 100 ml distilled water for 6 hours at slow heat. After every 2 hours, the samples were filtered through 8 layers of muslin cloth and then centrifuged at 6400 rpm for 15 minutes. Supernatants were collected in a flask and autoclaved at 121°C for 15 lbs pressure and then the samples were stored at 4°C until further use.

Ethanol extraction

All the plant materials were weighed ten grams and extracted in 100 ml ethanol (95%) kept on magnetic stirrer at 190–220 rpm for 24 hours. The mixtures were filtered through 8 layers of muslin cloth and the filtrates were centrifuged at 6400 rpm for 15 minutes. Supernatants were collected and solvent was evaporated. Extracts were stored at 4°C until further use.

Methanol extraction

All the materials were weighed ten grams and extracted in 100 ml methanol, and kept on magnetic stirrer at 190–220 rpm for 24 hours. The mixtures were filtered through eight layers of muslin cloth and then the filtrates were centrifuged at 6400 rpm for 15 minutes. Supernatants were collected and solvent was evaporated. Extracts were stored at 4°C until further use

Qualitative Phytochemical study

Phytochemical tests were carried out on the aqueous, ethanol, methanol extracts according to standard methods (Ajayi et al., 2011).

Test for Tannins

To about 1 ml of extract in a test tube a few drops of 0.1% ferric chloride was added and observed for brownish green or bluish black color development.

Test for Saponins (Foam test)

The presence of saponins was determined by Frothing test. To a small amount of extract in test tube, distilled water was added, shaken vigorously and allowed to stand for 10 minutes for stable persistent froth. Formation of froth confirms the presence of saponins (Kapoor et al., 1969).

Test for Flavanoids

To 1 ml of extract in the test tube few drops of dilute sodium hydroxide were added. An intense yellow color was observed in the test tube. Intense yellow color becomes colorless on addition of a few drops of dilute acid that indicated the presence of flavanoids.

Test for Terpinoids

2.5 ml of extract in test tube was followed by addition of 1 ml chloroform and concentrated sulphuric acid (1.5 ml) was carefully added to form a layer. A reddish brown coloration on interface was formed to indicate the presence of terpinoids.

Test for Alkaloids (Hager's test)

To small amount of extract, saturated solution of picric acid was added. The acid layer with Hager's reagent gave yellow precipitate indicating positive results for alkaloids.

Test for Glycosides (Keller-Kilani test)

To small amount of extract 2 ml of glacial acetic acid containing 1–2 drops of 2% solution of FeCl3. The mixture was then poured into another test tube containing 2 ml of concentrated sulphuric acid. A brown ring at the interphase indicated the presence of cardiac glycosides.

Determination of antimicrobial activity

The standard microbial strains were obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India. The bacterial strains were grown in their specific broth and maintained on agar slants at 4°C.

Antibiotics

Three antibiotics were used for antibiotic susceptibility study against standard microorganisms. All antibiotic discs were purchased from Hi-Media, Mumbai India. The names and concentration of the antibiotics used were as follows: Ampicillin (20 mg), Streptomycin (20 mg) and Penicillin (20 mg).

Disk Diffusion for Antimicrobial Susceptibility Testing

Whatman filter paper no. 1 was used to prepare discs approximately 6 mm in diameter, which were placed in beaker and sterilized in a hot air oven. The inoculum was prepared by making a direct broth or saline suspension of isolated colonies selected from 18 to 24 hour agar plate. The suspension was adjusted to match the 0.5 McFarland turbidity standards, using saline and a vortex mixer. After 16 to 18 hours of incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using a ruler, which was held on the back of the inverted petri plate.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the methanol plant extracts was determined using turbidimetric method. The microbial suspensions Escherichia coli, Staphlococcus aureus, Streptococcus mutans (Mcfarland standard) were inoculated in eppendorf tubes. To each eppendorf tube different volumes (650 µl to 700 µl) of Muller Hinton broth were added and to this sterile broth different concentration of Phyllanthus emblica, Terminalia chebula and Eucalyptus globulus methanol plant extract (1µl–50µl) was added. Finally, the eppendorf tubes were inoculated with 300 µl of microbial suspension under aseptic condition. Eppendorf tubes with broth and microbial suspension was used as a positive control. The least concentration of methanol plant extracts that inhibited the growth of bacteria was considered as minimal inhibitory concentration.

Statistical analysis

One-way ANOVA and Dunnet's multiple comparison test was performed to assess the statistical significance of antimicrobial activities of different plant extracts against the cariogenic microbes. A P value of 0.05 was taken as cut off and all statistical analyses were performed using Graphpad Prism version 6.04 (trial).

Results and Discussion

Preliminary qualitative phytochemical screening

The present study performed phytochemical screening on the extracts from the three plant sources to assess the presence of bioactive components. The presence of alkaloids, flavonoids, tannins, steroids, saponins were determined. The phytochemical characteristics observed are summarized in table 1.

Table 1: Preliminary qualitative phytochemical analysis of ethanol extracts of three plants Phyllanthus embilica, Terminalia chebula and Eucalyptus globulus

Compound	*Phyllanthus embilica*	*Terminalia chebula globulus*	*Eucalyptus*
Tannins	+	+	+
Saponin	-	-	+
Flavonoids	+	-	-
Terpenoids	+	+	+
Glycosides	-	+	+
Alkaloids	+	- +	

(+) present and (-) absent

The fruit extracts of Phyllanthus embilica were found to have tannins, flavonoids, terpinoids and alkaloids. Fruit extract of Terminalia chebula was found to have tannins, terpinoids and glycosides. Leaf extract of Eucalyptus globulus showed the presence of tannins, saponins, terpinoids, glycosides and alkaloids. Tannins and terpinoids were present in all the three plant extracts.

In the preliminary phytochemical screening, the methanol extract was positive for presence of tannins in all the plants. Tannins are known to possess anthelmintic activity. Presence of tannins and saponins in Phyllanthus emblica and Eucalyptus globulus may account for the antimicrobial effects exhibited by these plants on S. aureus, S. mutans and E. coli since these substances are known to have antimicrobial effects. Action of tannins may be due to protein denaturation and is found to be non-specific (Carter, 1986). Tannins in Phyllanthus emblica were reported to possess antioxidant activity (Gaire and Subedi, 2014). Flavonoids present in Phyllanthus emblica may inhibit RNA synthesis in microbes via intercalation of nucleic acid bases, contributing to its antimicrobial activity (Kumari and Khatkar, 2016).

Alkaloids present in Phyllanthus emblica and Eucalyptus globulus are known to exert antimicrobial activity by their ability to bind the DNA, thereby affecting replication and subsequent synthesis (Evans, 1992).

Eucalyptus globulus was also found to contain terpinoids. Terpinoids from some plants have been found to show anti HIV activity on different targets like reverse transcriptase, and protease (Cowan, 1999).

Antimicrobial activity and minimum inhibitory concentration

The antimicrobial activity of the extracts from the three plants varied greatly among the different solvents. Both the positive controls produced significant sized inhibition zones against the three test

bacteria (Ampicillin for gram-positive bacteria and Streptomycin for gram-negative bacteria). The nine extracts of three plants, screened for antimicrobial activity, showed antibacterial activity against S. mutans, S. aureus and E. coli. The methanol extract of Eucalyptus globulus was the most effective against S. mutans, S. aureus and E. coli. Methanol extracts of Eucalyptus globulus showed the highest zone of inhibition (12 mm) in S. aureus, followed by 10 mm in S. mutans, which was higher than the zone observed with Ampicillin (5 mm). Compared to aqueous and ethanol extracts, the methanol extracts of Phyllanthus emblica, Terminalia chebula and Eucalyptus globulus produced the largest zone of inhibition, among the extracts tested (Figure 1).

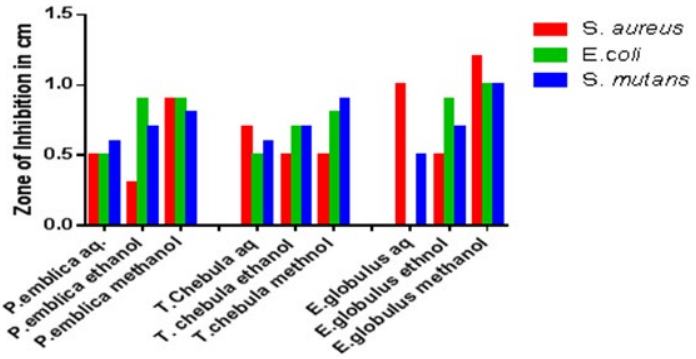

Figure 1: Comparison of zone of inhibition of aqueous, ethanol and methanol extracts of Phyllanthus emblica, Terminalia chebula and Eucalyptus globulus against S. aureus, E. coli and S. mutans

Based on preliminary screening assay, the methanol extracts of Phyllanthus emblica, Terminalia chebula and Eucalyptus globulus were further evaluated to determine the minimum inhibitory concentration (MIC). MIC was determined as the lowest concentration of the extract, which inhibited the growth of the tested microorganism. Based exhibited the profound and promising activity of Eucalyptus globulus.

MIC against S. aureus was observed at 100 µg/ml for Phyllanthus emblica, 100µg/ml for Terminalia chebula and at 500 µg/ml for Eucalyptus globulus methanol extracts. MIC against S. mutans was demonstrated at 500 µg/ml for Phyllanthus emblica, 100 µg/ml for Terminalia chebula and at 100 µg/ml for Eucalyptus globulus methanol extracts. MIC against E. coli was observed at 500 µg/ml for Phyllanthus emblica, 1.527µg/ml, 100 µg/ml for Terminalia chebula and at 100 µg/ml for Eucalyptus globulus methanol extracts.

The results of the MIC assay suggest that Phyllanthus emblica and Eucalyptus globulus were more effective against S. aureus when compared with Terminalia chebula. Eucalyptus globulus was more effective against S. mutans when compared with Terminalia chebula and Phyllanthus emblica. Phyllanthus emblica and Eucalyptus globulus were more effective against E. coli when compared with Terminalia chebula (Figure 2).

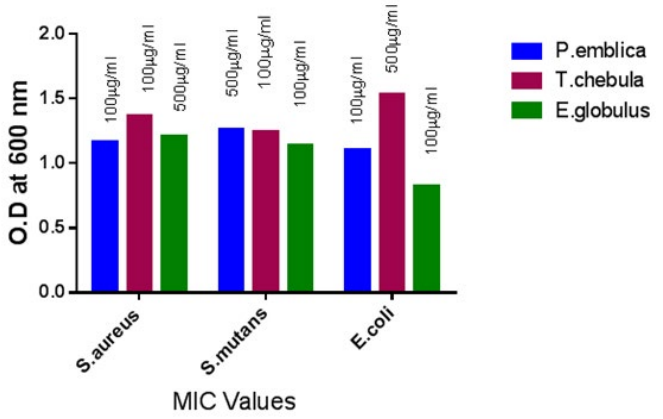

Figure 2: Graph representing minimum inhibitory concentration (MIC) values for different plant extracts against the cariogenic microbes tested in the study

These results indicate that the methanol extracts of plants screened had higher antimicrobial activity than the ethanol and aqueous extracts. Gram-positive microorganisms were more sensitive to the plant extracts than the gram-negative microorganisms.

The higher susceptibility of the gram-positive organisms to the plant extracts may be due to their cell wall structure, which is of a single layer while the gram-negative cell wall is a multi-layered structure and quite complex (Zampini et al., 2009; Nazzaro et al., 2013). The extracts evaluated in our study showed varying degree of antimicrobial activity on the microorganisms tested. The antimicrobial activity was more apparent in methanol than in ethanol extract of the same plant. This is supported by the study conducted by Eloff (1998) who reported that extraction with methanol resulted in better quantity and diversity of compounds from plants when compared with methanol.

Eucalyptus globulus was the most potent plant compared with Terminalia chebula and Phyllanthus emblica against the bacterial strains evaluated in our study. This study gives an indication of the efficiency of the plants obtained from traditional healers. The results from this study form a basis for further investigation into the potency of these plants, to isolate the compounds responsible for antimicrobial activity and results suggest that the plants tested may be a source of new antibiotic compounds.

Conclusions

The findings from the present study suggest that Phyllanthus emblica, Terminalia chebula and Eucalyptus globulus extracts might be effective as antimicrobial agents against oral pathogens. Methanol extracts of these plants revealed a promising MIC value against the cariogenic bacteria tested. The effects of this extract may be more beneficial if it is incorporated in gum, toothpaste, mouthwash and dental products to reduce plaque and dental caries.

Acknowledgements

We thank the dental department of Mahavir Hospital for discussions and for the samples for preliminary work, and Research Director of BMMRC for his encouragement. We thank Dr. Archana Jayraman for her help in critiquing the manuscript.

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