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ORIGINAL ARTICLE

Anticariogenic and phytochemical evaluation of *Eucalyptus globules* Labill.

Kalpesh B. Ishnava *, Jenabhai B. Chauhan [1], Mahesh B. Barad [2]

Ashok and Rita Patel Institute of Integrated Studies and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar-388121, Gujarat, India

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α-Farnesene

Abstract In the present study, *in vitro* anticariogenic potential of ethyl acetate, hexane and methanol and aqueous extracts of plant leaves of *Eucalyptus globules* Labill. were evaluated by using four cariogenic bacteria, *Lactobacillus acidophilus, Lactobacillus casei, Staphylococcus aureus* and *Streptococcus mutans*. Agar well diffusion method and minimum inhibitory concentration (MIC) were used for this purpose. The ethyl acetate extracted fraction of plant leaves showed good inhibitory effects against all selected bacteria. In *Eucalyptus globules*, hexane and ethyl acetate extracts found highly effective against, *Lactobacillus acidophilus* with MIC value of 0.031 and 0.062 mg/mL, respectively. Qualitative phytochemical investigation of above extracts showed the presence of alkaloids, phenolic compounds, steroids, cardiac glycosides and terpenes. Based on the MIC value and bioautography, ethyl acetate of plant leaf was selected for further study. Further investigation on the structure elucidation of the bioactive compound using IR, GC-MS and NMR techniques revealed the presence of alpha-farnesene, a sesquiterpene. *Eucalyptus globules* plant leaf extracts have great potential as anticariogenic agents that may be useful in the treatment of oral disease.

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1. Introduction

Dental caries is a multifactorial human disease that has widely affected many populations all over the world. According to WHO report on dental disorders, dental caries affects about 60% of the world adult population (Petersen, 2003). Until fairly recently, it was considered that early childhood caries, a particularly rampant form of caries manifested in young children, had a different etiology. There are mainly many different types of bacteria involved in dental caries process. Plaque is found preferentially at protected and stagnant surfaces, and these are at the greatest risk of disease (Scheie, 1994). Two major groups of bacteria produce such acids, namely, the mutans streptococci (including *Streptococcus mutans* and *Streptococcus sobrinus*) and the lactobacilli species (Loesche, 1986;

* Corresponding author. Tel.: +91-02692-229189(O), 09824918606(M).
E-mail addresses: ishnavakb203@yahoo.com (K.B. Ishnava), jbc109@yahoo.co.in (J.B. Chauhan), maheshbarad@yahoo.com (M.B. Barad).
[1] Phone: (O) + 91-02692-229189.
[2] Phone: (O) + 91-02692-229189.

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Leverett et al., 1993). *Streptococcus mutans* are the primary species associated with the early dental caries process (Loesche, 1986). There are undoubtedly other acidogenic organisms involved in dental caries. However, it is now obvious that the same bacteria are involved, but the reasons for the rapid progression of the disease in these children are still uncertain (Alaluusua et al., 1987; Caufield et al., 1993).

There are an overwhelming number of studies on the biological activities of plants and their natural product derivatives (Hebber et al, 2004; Cowan, 1999). It is well known to add to toothpastes and mouth rinses an extract of *Chamomille folia* in a dosage of 0.1-2.0% by weight (Kitagaki et al., 1983). This extract is anti-bacterial on micro biota of the mouth and can therefore be used against certain inflammations in the mouth. The increasing resistance to available antimicrobials has attracted the attention of the scientific community regarding a search for new cost-effective drugs of natural or synthetic origin (Pai et al., 2004; Fine et al, 2000). The *Eucalyptus* is used to control several diseases derived from microbial infections.

A *Eucalyptus globules* Labill. under the family of Myrtaceae. A large tree attains a height of 60 m or more with a straight, clean bole and smooth bark peeling off in long thin strips or sheets Leaves on young twigs opposite, sessile, cordate-ovate, glaucous gray; adult leaves alternate, lanceolate or ovate-lanceolate, acuminate, falcate. Flowers large, axillary, solitary or 2 to 3 together; calyx tube broadly turbinate, thick and woody. Fruits (capsules) semi-globular, containing numerous minute seeds (Pandey et al., 2005). It is reported to be antiseptic, astringent, deodorant, diaphoretic, expectorant, febrifuge, insect repellant, rubefacient. The bluegum eucalyptus is a folk remedy for abscess, arthritis, asthma, bronchitis, burns, cough, fever, flu, inflammation, leprosy, malaria, sores, sore throat and wounds (Duke and Wain, 1981; List and Horhammer, 1969–1979; Morton, 1981). Leaves contain 70–80% eucalyptol (cineol). Also includes terpineol, sesquiterpene alcohols, aliphatic aldehydes, isoamyl alcohol, ethanol, and terpenes (Morton, 1981). The main objective of the present study was to investigate the effects of leaf extracts of *Eucalyptus globules* for anticariogenic activity and phytochemical study.

2. Materials and Methods

2.1. Collection of plants material

Eucalyptus globules Labill. plant leaves were collected between January to February, 2010 from the surroundings of Vallabh Vidyanagar, Gujarat, India. The leaves of all the healthy and disease free plants were used to test the antibacterial activity. Plant specimens were identified by Dr. Kalpesh Ishnava (Plant Taxonomist) at Ashok and Rita Patel Institute of Integrated Study & Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, Gujarat, India.

2.2. Extraction of leaves

First of all the leaves of *E. globules* were thoroughly washed with tap water, blotted and dried under sunlight after cutting them into small pieces and subjected to oven drying at 60°C for 12 hours. For the purpose of making powder it was ground in a grinder. From this, 50 grams of powdered material was soaked in 250 mL of ethyl acetate for 24 hours at room temperature. The extract was filtered with the help of Whatman filter paper number-1. The filtrate was collected in petridish and dried at room temperature. The dried extract from petridish was scraped and transferred to eppendorf tube.

The residual material from the funnel was dried again and resuspended in 250 mL hexane for 24 hours at room temperature. The extract was filtered and collected in the petri dish. It was dried at room temperature.

Similarly, the residual materials from the funnel are preserved and reextracted with a same volume (250 mL) of methanol and distilled water respectively. In both cases, the resultant culture filtrate was air dried at room temperature. The dried extract from petri dish was scraped and transferred to eppendorf tube.

2.3. Cariogenic bacteria

A group of bacteria known to cause dental caries were selected and purchased from Microbial Type Culture Collection (MTCC) bank, Chandigarh as a freeze dried pure culture. The bacterial cultures were revived by using MTCC specified selective growth medium and preserved as glycerol stocks. The bacteria responsible for dental caries *Lactobacillus acidophilus* (MTCC-*447), *Lactobacillus casei* (MTCC-1423), *Streptococcus mutans* (MTCC-890) and *Staphylococcus aureus* (MTCC-96) were used for the study.

2.4. Inoculums Preparation

Fresh inoculums were prepared by streaking a loopful of bacterial suspension into the bacteria specific selective media (Himedia) and incubated at an optimal temperature in order to maintain an approximately uniform growth rate. The bacterial cultures were compared with 0.5 McFarland turbidity standard, which is equivalent to approximately $1X10^8$ bacterial cell count per mL (Perilla, 2003), was maintained throughout the experimentation.

2.5. Bioassay for anticarcinogenic activity of E. globules leaf extracts

2.5.1. Agar Well Diffusion Method

In the present study, to test anticariogenic activity, *E. globules* plant leaf extracts were used. The anticariogenic activity was studied by agar well diffusion method (Perez et al., 1990). From the stock, 100 mg of leaf extracts was suspended in one milliliter of each of ethyl acetate, hexane, methanol and distilled water. Selective agar medium plates were marked and divided in to 4 equal parts, labeled for specific organism and extract name. A fresh bacterial culture of 100μL having 10^8 CFU/mL was spread on agar plates with a glass spreader. A well of 10 mm diameter was punched off at previously marked petriplates into agar medium with sterile cup borer and then it was filled with 100 μL of *E. globules* leaf supernatant. Plates were placed for 30 minutes in a refrigerator for diffusion of extracts and then incubated at 37°C (or specified temperature) for 24 hours or more depending upon the bacterial species, until appearances of the inhibition zone. The zone of inhibition (including well diameter) was measured as a property of anticariogenic activity. Antibiotic, ampicillin was used as a standard at a concentration of 10 μg/mL and all the

organic solvents were used as positive control and negative control respectively. Bioassay was performed in duplicate and repeated twice.

2.5.2. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration was evaluated by the two fold serial broth dilution method (Chattopadhyay et al., 2001). Leaf extracts showing more than 10 mm inhibition zone were selected for MIC. Selective broth medium was used for dilutions as well as preparing inoculums. The bacterial cell density was maintained uniformly throughout the experimentation at $1X10^8$ CFU/mL by comparing with 0.5 McFarland turbidity standards. Leaf extracts of 100μL from the stock solution (10mg/mL) was taken into a first dilution tube containing 900μL of the selective medium broth and mixed well. From this, 500 μL were transferred to second tubes containing 500μL broth. This step was repeated nine times and from the last tube 500μL of the solution was discarded. 100μL of test organisms was added in each tube. The final volume of solution in each tube was made up to 0.6 mL. The MIC was tested in the concentration range between 10 μg/mL and 0.039 μg/mL. Tubes were incubated at an optimal temperature and time in an incubator. Growth indicator 2, 3, 5-Triphenyl tetrazolium chloride solution (100μL of 0.1%) was incorporated in each tube to find out the bacterial growth inhibition. Tubes were further incubated for 30 minutes under dark conditions. Bacterial growth was visualized when colorless 2, 3, 5-Triphenyl tetrazolium chloride was converted into red color formazan in the presence of bacteria. Each assay was repeated thrice by using DMSO and selective medium as control.

2.6. Phytochemical Characterization

2.6.1. Preliminary phytochemical analysis

Qualitative phytochemical analysis of *E. globules* crude leaf extracts selected based on MIC analysis was performed as per the standard methodology to determine the presence of Tannins, Alkaloids, Saponis, cardiac glycosides, Steroids, Terpenoides and Phenolic compounds (Parekh and Chanda, 2008).

2.6.2. Analytical thin layer chromatography

Analytical TLC was performed to find out a suitable solvent system for the development of chromatogram. Different solvent system was tried on precoated TLC plates (Merck, Silica gel 60 F254 plate, 0.25mm) for the development of chromatogram. Among all, Toluene: ethyl acetate: (93:7) solvent system was found to be the best and used for subsequent analysis.

2.6.3. Bioautography

By using capillaries 10μL of hexane extract of *E. globules* leaf extracts (100mg/mL stock solution) was spotted on to 0.25mm thick precoated silica gel 60 F254 plate (Merck, Germany). The band length was 2 mm thick. After air drying the TLC plate was run using pre-standardized solvent system, Toluene: ethyl acetate (93:7). The chromatogram was observed under UV illumination and used for bioautography. Specific growth medium (Tomato juice: 100 mL, Yeast extract: 5.0gm, Skimmed milk: 100gm per 1000 mL of distilled water, pH: 7.2), seeded with *Lactobacillus acidophilus*, was overlaid onto the silica gel plate loaded with sample and incubated at 37°C for 24 hrs. The next day, the plate was flooded with 2, 3, 5-Tri phenyl tetrazolium chloride (0.1%) to visualize growth inhibition. The area of inhibition zone appeared transparent against a red background (lawn of living bacteria).

2.6.4. Preparative thin layer chromatography (PTLC)

The preparative thin layer chromatography was performed at the final step of the purification of the pure compound prior to the structure elucidation. Bands that showed antibacterial activity were pulled together and further purified by preparative thin layer chromatography (PTLC). For the PTLC, sample aliquots were loaded onto TLC plates and developed in Toluene: ethyl acetate (93:7) solvent system. Bioautography of the TLC plate was used to confirm the position of the compound showing antibacterial activity. The compound was eluted from the developed plate by scrapping off silica gel and mixed well with hexane and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and used for further analysis.

2.6.5. Fourier Transformer Infra Red (FTIR) Spectroscopy

A thin film of *E. globules* plant leaf active eluted fraction in hexane was applied on the glass and IR spectra were recorded by using Perkin Elmer spectrophotometer, Spectrum Instrument (Germany) with FTIR paragon 1000 PC software at the Sophisticated Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidyanagar, Gujarat.

2.6.6. Gas Chromatography-Mass Spectroscopy (GC-MS)

The GC-MS analysis was done by the electron impact ionization (EI) method on Auto system XL gas chromatography (Perkin Elmer Instrument, Germany) coupled to a Turbo Mass Spectrophotometer (Perkin Elmer Instrument, Germany) at Sophisticated and Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidyanagar, Gujarat. The column was fused silica capillary column, 30 x 0.25 mm ID; coated with D-I, 0.25 μm film thickness. The temperature of the column was programmed at 70 to 250^0C at the rate of 10^0C/min increase, injection port temperature at 250^0C. Helium was used as carrier gas at a constant pressure of 100 kpa and a flow rate of 20mL/min. Samples which dissolved in chloroform were run fully at a range of 60-550 amu and the results were compared by using NIST 10^7 Spectral library search programme.

2.6.7. NMR Spectroscopy

^{1}H NMR spectra were recorded in $CDCl_3$ using a BRUKER and 400 MHz for proton NMR spectrometer at the Department of chemistry, Sardar Patel University, Vallabh Vidyanagar, Gujarat, India.

3. Result and discussion

The results of anticariogenic activity of the extracts and their efficacy are quantitatively assessed by the presence or absence of the zone of inhibition and diameter (in mm) respectively (Table 1). Four different solvents were used for the extraction of anticariogenic substances (Table 1). The solvents used were hexane, ethyl acetate, methanol and distilled water. Among all

the solvents, ethyl acetate proved to be the most prominent solvent for the extraction of anticariogenic substances from the selected plant.

Distilled water extract of *E. globules* leaf extracts is only active against *L. acidophilus*. Ethyl acetate extracts are active against all the selected bacterial strain. Maximum activity against *L. acidophilus, S. mutans* and *L. casei* recorded with the zone of inhibition is 13mm, 13mm and 11 mm respectively (Table 1). Hexane extracts of *E. globules* are active against all selected microorganisms. The maximum zone of inhibition against *L. acidophilus, S. mutans* and *L. casei* is 12mm, 8mm and 10 mm respectively (Table 1). The maximum activity of methanolic extract of plant leaves against *L. acidophilus, L. casei* and *S. mutans* is 12mm, 12mm and 13 mm respectively (Table 1) showed. Bachir and Mohamed reported *E. globules* activity against *Staphylococcus aureus* Gram (+) and *Escherichia coli* Gram (-) bacteria (Bachir and Mohamed, 2008). Sirivan Athikomkulchai reported the volatile oils showed good antimicrobial activity against *Propionibacterium acnes* (MIC, MBC = 9.38 mg/ml for eucalyptus oil) (Sirivan et al., 2008). Methanolic extract of *Eucalyptus globules* leaves is very much effective in comparison to those which were carried out by Kachhiya (2008) on *E. globules* methanolic extract of *E. globules* stem on selected anticariogenic bacteria (Kachhiya, 2008).

The Minimum Inhibitory Concentration (MIC) values of leaf extracts of all the selected organic solvents showing the highest activity against selected bacteria are assessed and summarized in Table 1. The maximum MIC value was found to be 2 mg/mL and the minimum value as 0.031 mg/mL (Table 1). The MIC value of hexanolic extract of *E. globules* against LA and LC was 0.062 mg/mL and 0.25 mg/mL respectively (Table 1). The MIC value of ethyl acetate extract of *E. globules* was most significant against LA i.e 0.031 mg/mL and that against LC as 0.25 mg/mL (Table 1). As compared to above solvents, methanolic extracts exhibited MIC values ranging from 0.5 to 2 mg/mL against selected cariogenic bacteria (Table 1).

The presence of common phytochemical constituents such as alkaloids, tannins, saponins, terpenoids, steroids, phenolic compounds and cardiac glycosides was tested qualitatively as per the methodology of Ahmad and Beg (2001) (Table 2). The bioactive compound found in the hexane extract of *E. globules* includes alkaloids, terpenoids, steroids, phenolic compounds and cardiac glycosides (Table 2). A similar observation was made in the ethyl acetate extract of *E. globules* except there is absence of alkaloids (Table 2). Methanolic extracts of *E. globules* also exhibited similar phytochemical profile.

Table 1 Anticariogenic activity of extracts of *Eucalyptus globules* leaves.

Plant extracts	Zone of Inhibition in mm (MIC in mg/ml)			
	L. acidophilus	***L. casei***	***S. aureus***	***S. mutans***
Distilled water	03	-	-	-
Ethyl acetate	13(0.033)	11(0.25)	04	13
Hexane	12(0.062)	10(0.25)	04	08
Methanol	12(01)	12(0.5)	08	13(02)
Cefadroxil	36	31	31	12
Erythromycin	23	15	19	15
Tetracycline	28	31	24	28

Table 2 Phytochemical analysis of *Eucalyptus globules* leaves extracts.

Phytochemical Test	1	2	3	4	5	6	7
E. globules(Hexane)	-	-	+	+	+	+	+
E. globules (Ethyl acetate)	-	-	+	+	+	+	-
E. globules (Methanol)	+	+	+	+	+	+	-

1-Tannins, 2-Saponins, 3-Cardiac glycosides, 4-Steroids, 5 –Terpenoids, 6-Phenolic compounds, 7-Alkaloids, Absent = (-), Present = (+)

In order to find out the active principles present in *E. globules* of hexane, ethyl acetate and methanolic extracts, TLC solvent system was standardized (Toluene: Ethyl acetate (93:7)) and used for subsequent analysis. The bioactive compounds were separated from crude extracts by using TLC technique.

To locate the major active compounds responsible for the anticariogenic activity in *E. globules*, chromatogram was used for TLC – bioautography against SMU, LA and LC.

The UV analysis of TLC plate of *E. globules* crude hexane extracts showed orange fluorescence bands at 254 nm. Red and blue fluorescence bands were observed in hexane extracts of *E. globules* at 365 nm. *E. globules* ultraviolet spectra of hexanolic extracts of various intensities are plotted in Fig. 1. Spectral analysis shows the total nine numbers of active bands with R_f Value 0.32. The chromatogram was used for bioautography against *Lactobacillus acidophilus*.

The analysis of TLC plate run from eluted sample showed red fluorescence at 254 nm and blue florescence at 366 nm, respectively, the single band was confirmed by using iodine vapor. The study of infrared spectra (IR) revealed the presence of H-bonded, Alkene C = C, NO_2 Nitro group, C–N stretch vib aliphatic, Alkene bending vib and C-Cl chloride as a major functional group (Fig. 2). The peak showing a maximum percentage area at RT 17.75 in GC–MS analysis and scan 1.87e4 through mass spectrophotometer and NMR, revealed the presence of alpha-farnesene ($C_{15}H_{24}$) and has a molecular weight

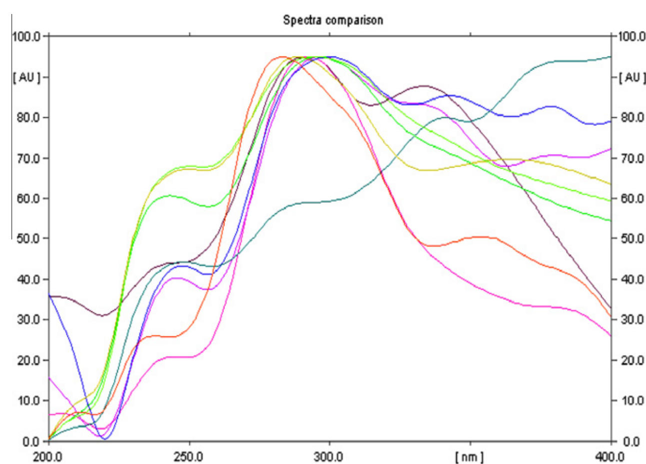


Figure 1 Ultraviolet spectra of various intensities from hexanolic leaf extract of *E. globules*.

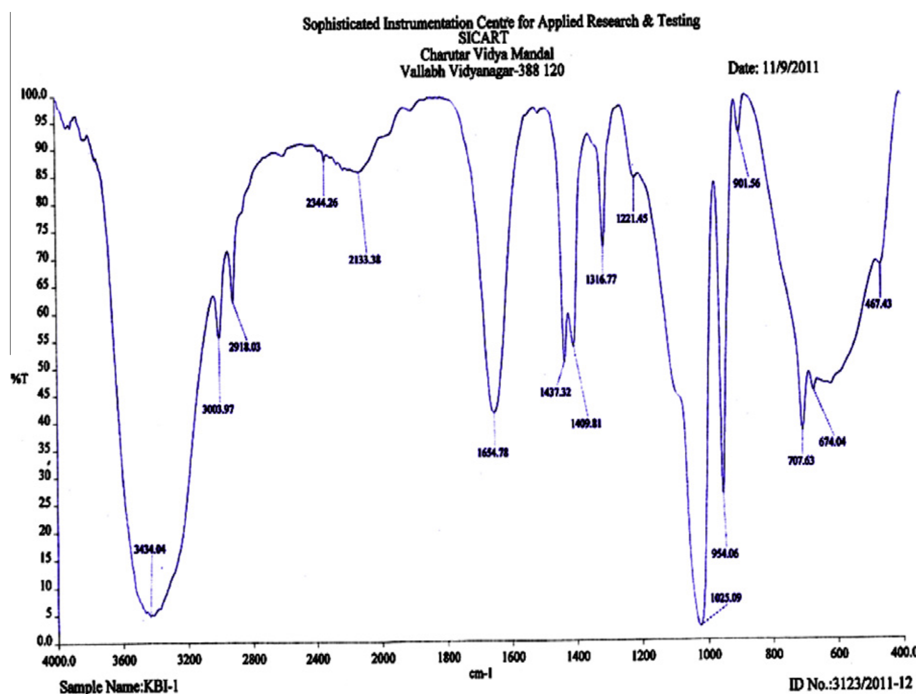
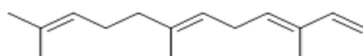


Figure 2 IR spectra of crude leaf extract (in Hexane) of Eucalyptus globules.

of 204.35, pK is 17.75 (Fig. 2). The compound identified as alpha-farnesene, a sesquiterpene (Fig. 3). Claudia et al (2003) reported a similar type of compound in different types of chopped leaves of *Eucalyptus dunnii, E. citriodora*, and *E. saligna* (Claudia et al., 2003). It is also an active compound present in the leaf oils of *E. globules* a taxonomically very close species.

4. Conclusion

Our findings on a broad spectrum activity of various extracts of *E. globules* against a panel of selected cariogenic bacteria and its phytochemical analysis revealed that it has an anticariogenic substance, one is identified as alpha-farnesene, a sesquiterpene. The good inhibitory potential of hexanolic and ethyl acetate extracts of *E. globules* plant leaves against *Lactobacillus acidophilus* and a panel of cariogenic bacteria will be useful in the future development of effective formulations of drugs for the control of dental caries.



General Name: alpha-Farnesene

Chemical Name: Farnesene

Chemical Formula: $C_{15}H_{24}$

Molecular Weight: 204.35

Figure 3 Structure of anticariogenic compound from *E. globules* active against cariogenic bacteria *Lactobacillus acidophilus (*Peak 17.75 of GC-MS).

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