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Original Research

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Anticariogenic activity and phytochemical studies of crude extract from some Indian plant leaves

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ABSTRACT

Aim: The aim was to screen the selected Indian plants for their antibacterial efficacy against four cariogenic bacteria *Lactobacillus acidophilus* (LA)(Microbial Type Culture Collection [MTCC]-*447), *Lactobacillus casei* (LC) (MTCC-1423), *Streptococcus mutans* (SMU) (MTCC-890) and *Staphylococcus aureus* (MTCC-96). To identify and characterize active principle present in these plants for the treatment of dental caries. **Materials and Methods:** The dried plant leaves materials are extracted by cold extraction using hexane, ethyl acetate, methanol, and distilled water. The solvents were evaporated, and the dried masses were suspended in dimethyl sulfoxide and used for anticariogenic activity by agar well diffusion method. Minimum inhibitory concentration (MIC) was evaluated by two-fold serial broth dilution method. Preliminary phytochemical analysis of effective extract was carried out by thin-layer chromatography (TLC) and bioautography. **Results:** Ethyl acetate and hexane extract of *Eucalyptus globules* was found most effective against *L. acidophilus* with MIC value 31 μg/ml and 62 μg/ml, respectively. Ethyl acetate extracts of *Acacia nilotica* and methanolic extract of *E. globules* also exhibited antibacterial activity against SMU and *L. casei* with MIC value of 50 μg/ml. Qualitative analysis of *E. globules* revealed the presence of alkaloids, terpenoids, phenolic compounds, and cardiac glycosides. The active principle responsible for the anticariogenic activity from *E. globules* were separated by TLC and subjected to bioautography using SMU, LA and LC. **Conclusion:** Anticariogenic activity and preliminary phytochemical analysis revealed that *E. globule* have potential to treat dental caries.

KEY WORDS: Anticariogenic activity, dental problem, leaves, phytochemical analysis

INTRODUCTION

Plants have always been a source of medicines. Plants produce the diverse range of bioactive molecules from its different parts such as leaves, stem, latex, bark, root, flower, and seeds, known as secondary metabolites that are involved in plants defenses mechanism against microorganisms, insects and herbivores. Bioactive molecules found in plants are tannins, alkaloids, saponins, cardiac glycosides, steroids, terpenoids, flavinoids, phenolic compounds, and many more [1].

Dental caries is an infectious disease that damages the structures of the teeth. Tooth decay or cavities are consequences of caries. If left untreated the disease can lead to severe pain and infection in severe cases can cause tooth loss. The pH of a healthy mouth is between 6.2 and 7.0. When the pH is <5.5 the tooth is in an acid environment and demineralization of the tooth occurs. The tooth is now in an acid environment and starts to demineralization. As the enamel loses its minerals, it starts to break down, resulting in the formation of a cavity [2]. Sticky foods are more harmful than non-sticky foods because they remain on the surface of the teeth. Tooth decay is caused by certain types of acid-producing bacteria (specifically *Lactobacillus acidophilus* [LA] [Microbial Type Culture Collection (MTCC)-*447], *Lactobacillus casei* [LC][MTCC-1423], *Streptococcus mutans* [SMU][MTCC-890] and *Staphylococcus aureus* [SA][MTCC-96]) which cause damage in the presence of fermentable carbohydrates such as sucrose, fructose, and glucose [3]. Oral *Streptococci*, which are major members of oral flora, frequently cause bacteremia and infective endocarditis [4]. SMU, a major causative agent for dental caries, has occasionally been isolated from the blood of patients with infective endocarditis [5,6]. Chung *et al*. identified four different SMU strain from 522 different streptococcal isolates, which were derived from patient's infectious endocarditis, sepsis and bacteremia following biochemical, serological and genetic analyses [3].

As the activity of plants extracts varies against different oral bacteria, the screening for antimicrobials from plants is a feasible approach to the identification of natural compounds with antimicrobial properties against dental pathogens [7,8]. 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 [7,9]. In India, particularly Gujarat state is a rich source of medicinal plants. About 750 species of medicinal plants are being used by tribal peoples residing in the remote areas [10]. There are many reports on the antibacterial activity of medicinal plants from India [11-13], but there is meager information specifically against cariogenic bacteria. Therefore, the present study was undertaken to screen and characterize selected ethnobotanically important plant extracts for their efficacy to that dental caries or tooth diseases.

MATERIALS AND METHODS

Plant Materials

Plant species were collected between January and February, 2010 from different parts of Gujarat and surroundings of Vallabh Vidyanagar [Table 1]. The leaves of all the healthy and disease free plants were used to test the antibacterial activity. The plant specimens were identified by Dr. Kalpesh Ishnava (plant taxonomist) at Ashok and Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, Gujarat, India.

Preparation of Plant Leaves Extracts

First of all, the leaves of respective plants were thoroughly washed with running tap water, blotted and dried under sunlight. The leaves were then ground to powder in a grinder (Maharaja Mixer Ltd). The powdered materials (50 g) were soaked in 250 ml of hexane for 24 h at room temperature under shaking condition (130-140 rpm). The extract was then filtered with the help of Whatman filter paper number-1, collected in the petri dish and dried at room temperature. The dried extract from petri dish was scraped and transferred to Eppendorf tube.

Table 1: Details of plants selected

Plant name	Family	Local name	Collection site
Ficus racemosa L.	Moraceae	Umardo	V. V. Nager
E. globules Labill.	Myrtaceae	Nilgari	Karamsad
A. indica A. Juss.	Meliaceae	Limado	V. V. Nager
M. zapota (L.) van Royen	Sapotaceae	Chiku	Jetpur
P. granatum L.	Punicaceae	Dadam	Jetpur
C. papaya L.	Caricaceae	Pappaya	Jetpur
T. patula L.	Asteraceae	Marigold	Dakor
Murraya koenigii (L.) Spr.	Rutaceae	Mitholimado	Vadodara
T. peruviana (Pers.) Merr.	Apocynaceae	Pidikaren	New V. V. Nager
N. tabacum L.	Solanaceae	Tamaku	New V. V. Nager
A. nilotica (L.) Del.	Mimosaceae	Bavad	Karamsad
Cordia gharaf (Forsk.) E. & A.	Ehretiaceae	Gudao	New V. V. Nager
Nyctanthes arbortristis L.	Oleaceae	Parijatak	Vadodara
Lantana camaravar. aculcata (L.) Mold.	Verbenaceae	Gathathi	New V. V. Nager
Anthocephalus cadamda	Rubiaceae	Kadam	New V. V. Nager
A. occidentale L.	Anacardiaceae	Kaju	New V. V. Nager
Alangium salvifolium (L. f.) Wang.	Alangiaceae	Ankol	New V. V. Nager
Lomonia acidissima L.	Rutaceae	Kothu	Karamsad
Ocimum basilicum L.	Labiatae	Damaro	New V. V. Nager
E. nivulia Buch.-Ham.	Euphorbiaceae	Thor	Karamsad

E. globules: Eucalyptus globules, A. nilotica: Acacia nilotica, C. papaya: Carica papaya, P. granatum: Punica granatum, T. patula: Tagetes patula, E. nivulia: Euphorbia nivulia, N. tabacum: Nicotiana tabacum, A. indica: Azadirachta indica, M. zapota: Manilkara zapota, T. peruviana: Thevetia peruviana, A. occidentale: Anacardium occidentale

The residual material from the funnel was dried again and resuspended in 250 ml ethyl acetate for 24 h at room temperature under shaking condition (130-140 rpm). The extract was filtered and collected in the petri dish. It was dried at room temperature. Similarly, the residual materials from the funnel were preserved and re-extracted with the same volume (250 ml) of methanol and then distilled water respectively. In both cases, culture filtrates were air dried at room temperature. The dried extracts from petri dish were scraped and transferred to Eppendorf tube and used for analysis.

Cariogenic Bacterial Strains

A group of bacteria known to cause tooth decay were selected and procured from 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 cariogenic bacteria used in the present study includes *L. acidophilus* (MTCC-*447), *L. casei* (MTCC-1423), SMU (MTCC-890) and *S. aureus* (MTCC-96).

Preparation of Inoculums

Fresh bacterial cultures were prepared by streaking loopful of bacterial suspension in to organism specific selective media (Hi-media) and incubated at optimal temperature for growth. The isolated bacterial colonies from freshly grown media plates were then inoculated to bacteria specific media and growth were compared with 0.5 McFarland turbidity standard, which is equivalent to approximately 1×10^8 bacterial cells count/ml was maintained throughout the experimentation [14].

Screening for Anticariogenic Activity

Agar well diffusion method

In the present study, to test anticariogenic activity, 20 different plant extracts were used. The anticariogenic activity was studied by agar well diffusion method [15]. From the stock, 100 mg of each plant extract were suspended in 1 ml of dimethyl sulfoxide (DMSO). The agar plates were prepared and incubated overnight at 37°C. Agar plates were marked and divided in to four equal parts, labeled for specific bacteria and extract. A fresh bacterial culture of 100 μL having 10^8 CFU/ml was spread on agar plates with glass spreader. A well of 10 mm diameter punched off at previously marked petri plates into agar medium with sterile cup borer and then it was filled with 100 μL of respective plant leaves extracts. Plates were placed for 30 min in refrigerator for diffusion of extracts and then incubated at 37°C (or specified temperature) for 24 h or more depending upon the organisms, until appearances of zone of inhibition. The zone of inhibition (excluding well diameter) was measured as a property of anticariogenic activity. Antibiotics, cefadroxil,

erythromycin and tetracycline were used at concentrations of 100 μg/ml and 100% DMSO were used as positive and negative control, respectively. Bioassay was performed in duplicate and repeated twice [16].

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by the two-fold serial broth dilution method [16]. Plant extracts showing >9 mm inhibition zone were selected for MIC determination. Selective broth medium was used for dilutions as well as inoculums preparation. The bacterial cell density was maintained uniformly throughout the experimentation at 1×10^8 CFU/ml by comparing with 0.5 McFarland turbidity standards. Forty microliter of plant extracts from a stock (100 mg/ml) was taken into first dilution tube containing 960 μL of selective medium broth and mixed well. From these, 500 μL were transferred to second tubes containing 500 μL broths. This step was repeated 9 times and from the last tube 500 μL solutions was discarded. The 100 μL of test bacteria was added in each tube. The final volumes of solution in each tube were made up to 0.6 ml. The MIC was tested in the concentration range between 4.0 and 0.0031 mg/ml. The tubes were incubated at optimal temperature and time. Growth indicator 2,3,5-triphenyltetrazolium chloride solution (100 μL of 0.1%) was added in each tube to find out the bacterial growth inhibition. Tubes were then incubated for 30 min under dark condition. Bacterial growth was visualized when colorless 2,3,5-triphenyltetrazolium chloride converted into red color formazone in the presence of bacteria. Each assay was repeated thrice by using DMSO and selective medium as control.

Preliminary Phytochemical Analysis

Qualitative phytochemical analysis of all the plant leaves extracts selected, based on MIC value were performed as per the methodology [13].

Thin-Layer Chromatography (TLC)-Bioautography

Of 20 plants leaves extracts tested for anticariogenic activity. Only one plant extracts was selected showing maximum growth of inhibition against SMU, *L. casei* and *L. acidophilus* were respectively selected for used for bioautography. By using capillaries 10 μL of aqueous extract of *Eucalyptus globules* leaves (100 mg/ml stock solution) was spotted onto 0.25 mm thick pre-coated silica gel 60 F254 plate (Merck, Germany). The band length was 2 mm thick. After air drying, the TLC plate was run in pre-standardized solvent system, toluene:ethyl acetate (93:7). The chromatogram was observed under ultraviolet (UV) illumination and used for bioautography. Agar medium seeded with specific bacteria SMU, *L. casei* and *L. acidophilus* was overlaid onto the above silica gel plates and incubated at 37°C for 24 h. Next day, the plate was flooded with 2,3,5-triphenyltetrazolium chloride (0.1%) to visualize growth inhibition. The areas of inhibition zone were appeared as transparent zone against reddish background (lawn of living bacteria).

RESULTS

The results of anticariogenic activity of the plants extracts and their efficacy are quantitatively assessed by recording the presence or absence of zone of inhibition and diameter, respectively. Four different solvents were used for the extraction of anticariogenic substances [Table 2].

Only 45% of plants give rise to anticariogenic substances out of 20 selected plants, as extracted with hexane and tested against for selected cariogenic bacteria (SMU, LA, LC, and LA) [Table 2]. Among them, *E. globules* was found to be active against all the four selected cariogenic bacteria, with maximum (12 mm) zone of inhibition in LA, followed by LC (10 mm), SMU (8 mm) and SA (4 mm).

Fourteen (i.e., 70% plants) of 20 selected plants demonstrated broad spectrum of anticariogenic activity against four selected bacteria, when ethyl acetate was used for extraction of anticariogenic substances [Table 2]. Among these, three plants viz., *E. globules*, *Acacia nilotica* and *Anacardium occidentale* were found to be active against all the four selected cariogenic bacteria, in which *E. globules* showed maximum zone of inhibition (13 mm) against LA. A. *nilotica* and A. *occidentale* gave 12 mm zone of inhibition in SMU and in LC, respectively.

There was an equal ratio of plants that did not show the anticariogenic activity, when methanolic extracts of 20 selected plants were evaluated [Table 2]. *E. globules* and *Manilkara zapota* were found to be active against all the four selected cariogenic bacteria. Here, *E. globules* showed maximum and equal zone of inhibition against SMU, LA, and LC (12 mm), followed by A. *occidentale* against LC (12 mm) and *Punica granatum* against LC (11 mm).

Finally, when distilled water was used as a solvent for extraction of anticariogenic substances and tested, 30% of plants extracts exhibited anticariogenic activity against SMU, LC, LA, and SA [Table 2]. The rest of the plants extracts (70%) doesn't showed anticariogenic activity.

MIC Values of Selected Plant Extracts

The MIC values of all the plant extracts showing highest anticariogenic activity against selected bacteria are presented in Figure 1. Examining the MIC values of nine samples of various extracts generated the data where the maximum MIC value was found to be 200 μg/ml and the minimum value as 31 μg/ml [Figure 1].

Bioautography Study

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

Table 2: Anticariogenic activity of different solvent extracts (in mm)

Plant name	Hexane				Ethyl acetate				Methanol				Distilled water			
	SMU	LA	LC	SA	SMU	LA	LC	SA	SMU	LA	LC	SA	SMU	LA	LC	SA
Ficus racemosa	-	-	-	-	-	10	-	-	-	07	-	-	-	-	-	-
E. globules	08	12	10	04	11	13	11	04	12	12	12	08	-	-	-	-
A. indica	05	05	-	-	05	-	-	-	07	-	-	-	-	03	-	-
M. zapota	-	07	-	-	04	-	-	-	08	08	06	08	05	-	-	06
P. granatum	06	-	-	-	09	-	08	-	10	-	11	08	05	-	-	-
C. papaya	04	07	-	-	05	07	-	-	-	05	-	03	-	-	-	06
T. patula	07	08	-	-	05	10	-	-	05	10	-	-	-	-	-	-
Murraya koenigii	-	-	-	-	-	07	-	-	-	-	-	-	-	-	-	-
T. peruviana	03	-	-	-	04	-	-	-	-	-	-	-	-	-	-	-
N. tabacum	03	-	-	-	05	-	-	04	-	-	-	-	-	-	-	-
A. nilotica	-	-	-	-	12	11	08	07	09	10	06	-	-	-	-	-
Cordia gharaf	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nyctanthes arbortristis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. camara	-	-	-	-	-	12	-	-	-	-	-	-	-	-	-	-
Anthocephalus cadamda	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. occidentale	-	-	-	-	09	08	12	08	10	-	12	08	-	07	-	-
Alangium salvifolium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lomonia acidissima	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ocimum basilicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. nivulia	04	-	-	05	08	-	02	-	06	-	05	06	-	-	02	07
Antibiotics																
Tetracycline	28	28	41	26	28	28	41	26	28	28	41	26	28	28	41	26
Cefadroxil	12	36	41	31	12	36	41	31	12	36	41	31	12	36	41	31
Erythromycine	15	23	19	19	15	23	19	19	15	23	19	19	15	23	19	19
DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

SMU: *Streptococcus mutans*, LA: *Lactobacillus acidophilus*, LC: *Lactobacillus casei*, SA: *Staphylococcus aureus*, DMSO: Dimethyl sulfoxide, *E. globules: Eucalyptus globules, A. nilotica: Acacia nilotica, C. papaya: Carica papaya, P. granatum: Punica granatum, T. patula: Tagetes patula, E. nivulia: Euphorbia nivulia, N. tabacum: Nicotiana tabacum, A. indica: Azadirachta indica, M. zapota: Manilkara zapota, T. peruviana: Thevetia peruviana, L. camara: Lantana camara, A. occidentale: Anacardium occidentale*

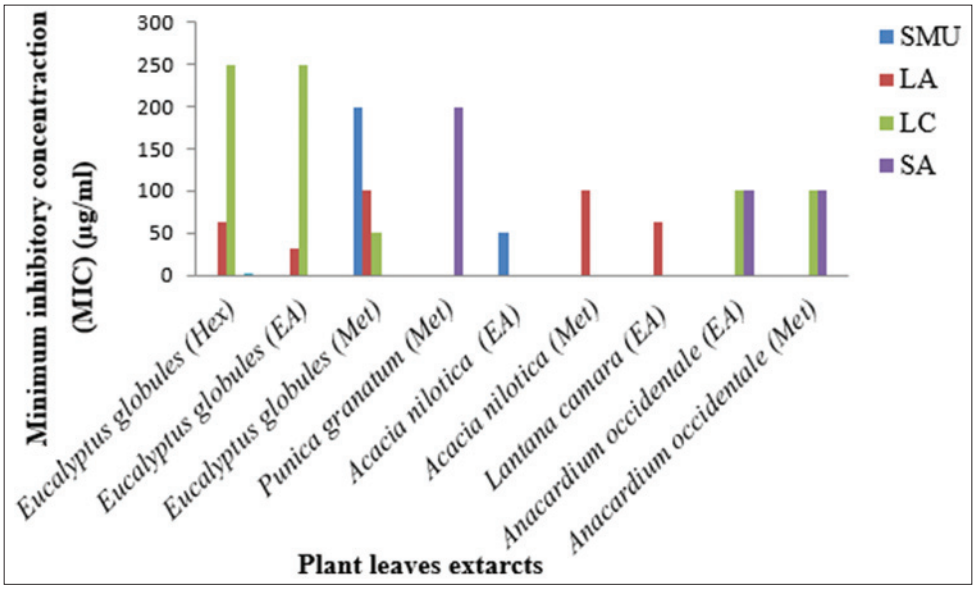

Figure 1: Minimum inhibitory concentration (µg/ml) of selected plant leaves extracts against cariogenic bacteria

chromatogram was observed under UV illumination and based on the different fluorescence color identified the particular phytochemical constituents of the plant extracts. The presence of common phytochemical constituents such as alkaloids, tannins, saponins, terpenoids, steroids, phenolic compounds, and cardiac glycosides were tested qualitatively as per the methodology [12] and presented in Table 3. The bioactive compounds found in hexanolic and ethyl acetate extracts of *E. globules* showed almost same constituents (terpenoids, steroids, phenolic compounds and cardiac glycosides), except alkaloid, which was present in the hexanolic extract. Methanolic extract of *E. globules* showed the presence of all the tested compounds except alkaloids [Table 3]. To identify the major active compounds responsible for the anticariogenic activity in

Table 3: Phytochemical constituent in various solvent extracts

Plant name	1	2	3	4	5	6	7
E. globules (Hex)	-	-	+	+	+	+	+
E. globules (EA)	-	-	+	+	+	+	-
E. globules (Met)	+	+	+	+	+	+	-
P. granatum (Met)	+	+	-	-	-	+	+
A. nilotica (EA)	+	+	-	-	-	+	+
A. nilotica (Met)	+	+	-	-	-	+	+
L. camara (EA)	-	-	-	-	-	-	+
A. occidentale (EA)	-	-	-	+	-	-	+
A. occidentale (Met)	+	+	-	+	-	+	+

-: Absent, +: Present, 1: Tannins, 2: Saponins, 3: Cardiac glycosides, 4: Steroids, 5: Terpenoids, 6: Phenolic compounds, 7: Alkaloids, *E. globules: Eucalyptus globules, A. nilotica: Acacia nilotica, P. granatum: Punica granatum, L. camara: Lantana camara, A. occidentale: Anacardium occidentale*

E. globules, chromatogram was subjected to TLC-bioautography against SMU, LA, and LC. The UV analysis of TLC plate of *E. globules* crude hexane and ethyl acetate extracts showed orange fluorescence bands at 254 nm (low intensity). Red and blue fluorescence bands in hexane at 365 nm (high intensity) whereas green, red, and blue bands were observed in ethyl acetate fraction.

DISCUSSION

Recently, antimicrobial properties of plants are being increasingly reported from all parts of the world because of emergence of multiple drug resistance of modern pharmaceuticals to human pathogenic organisms [12]. The compound present in the plants either inhibits the growth of microbial pathogen or kill them and have no toxicity to host cells are considered for developing new antimicrobial drugs. Different parts of plants supplying low cost medicine to human population have been used in Indian traditional system for the treatment of various human diseases. Natural products have been used to prevent oral diseases, especially plaque-related diseases, such as dental caries [17]. Our result of crude extracts of hexane, ethyl acetate and methanol of *E. globules*, *A. nilotica*, *L. camara*, *A. occidentale* and *P. granatum* showed very good anticariogenic activity against *L. casei*, SMU, *L. acidophilus* and *S. aureus*. Therefore, they are subjected to MIC determination and preliminary phytochemical analysis.

In the present study, ethyl acetate was found to be the most effective solvent for the extraction of anticariogenic substances from the selected plants. Hexanolic extract of *Azadirachta indica*, *Carica papaya*, and *Tagetes patula* were found active against both SMU and LA. Hexanolic extracts of 55% of the plants did not show any activity. Ethyl acetate extract of *P. granatum*, *C. papaya*, *T. patula*, *Nicotiana tabacum* and *Euphorbia nivulia* were found to be moderately active against selected bacteria. The extracts of *T. peruviana*, *N. tabacum* and *E. nivulia* found to be least active when tested against SMU and LC with 2-4 mm zone of inhibition. Slight zone of inhibition was observed in *E. globules* and *M. zapota* against SA and SMU respectively. Ethyl acetate of the six plants (i.e., 30%) did not show any anticariogenic activity. Bothelo *et al*., 2007 studied antimicrobial activities of essential oils from *Lippia sidoides* against oral pathogens responsible for dental caries and found that SMU was the most sensitive. Methanolic extract of *P. granatum*, *A. occidentale* and *A. nilotica* were found active against all the selected bacteria with varied zone of inhibition ranging from 6 to 12 mm. The extracts *C. papaya* and *T. patula* were active against SMU, LA and SA (3-5 mm). In this study, *E. globules* methanolic extract was found to be more effective than was reported [18], where, she used methanolic extract of *E. globules* stem. Aqueous extracts of *E. nivulia* and *A. occidentale* showed the highest (7 mm) activity against LA and SA, respectively (7 mm). *A. indica*, *M. zapota* and *P. granatum*, showed very little activity against all the selected anticariogenic bacteria. The lowest zone of inhibition was observed in *E. nivulia* (2 mm) against LC. The aqueous extracts are totally different compare to the other work where they reported that aqueous extracts are highly effective than hexane, ethyl acetate, and methanol [11].

The MIC values of hexanolic extract of *E. globules* against LA and LC was 62 μg/ml and 25 μg/ml, respectively [Figure 1]. The MIC value of ethyl acetate extract of *E. globules* was found most effective against LA (31 μg/ml) and that against LC (250 μg/ml). The MIC value of *A. nilotica*, *L. camara*, and *A. occidentale* was determined to be 500 μg/ml for SMU, 62 μg/ml for LA and 1 00 μg/ml for LC and LA [Figure 1].

Development of TLC plate by spraying anisaldehyde - sulfuric acid reagent showed strong blue colored bands, which indicates that terpenoids, phenylpropanoids, or saponins may be present. TLC plate when sprayed with vanillin – sulfuric acid reagent showed dark blue colored bands which depicts that essential oils viz., terpenoids, phenylpropenoids, or saponins might be present [Table 3]. Sulfuric acid (10%) when sprayed showed colored band indicating presence of cardiac glycosides [Table 3]. Ahmad and Beg reported broad spectrum of antibacterial activity of *E. globules* and also found presence of alkaloids, phenol and tannins when extracted with ethyl alcohol [12].

CONCLUSION

India has the richest diversity in the world in regards to resources of medicinal plants. The increasing failure of chemotherapeutics and antibiotics resistance exhibited by pathogenic microorganisms has led to the screening of several medicinal plants for their potential antimicrobial activity. Our findings on the broad spectrum activity of various extracts of *E. globules* against a panel of selected cariogenic bacteria and its primary phytochemical analysis revealed that it has anticariogenic substances. Chromatographic and spectroscopic analysis of *E. globules* extracts helped us to identify novel anticariogenic compounds.

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