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Anticariogenic Activity and Phytochemical Studies of Crude Drug Extract of Some Indian *Plants* for Dental Care

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Abstract
Oral diseases are a major health problem worldwide. The increasing failure of chemotherapeutics and antibiotics resistance exhibited by pathogenic micro-organisms has led to the screening of several medicinal *plants* for their anticariogenic activity [1]. The present study was undertaken to evaluate the anticariogenic activity of some folklore *plants* for dental health. Argemone mexicana, Pongamia pinnate, Solanum virginianum and Thevetia peruviana were tested against the bacteria that cause dental carries. Ethanolic extracts were prepared by maceration and soxhlet methods and they studied for phytoconstituents by chemical tests. A. mexicana seed extract produced greater zone of inhibition when compared other extracts. The MIC of A. mexicana seeds extract was found to be lower Hence; it was decided to study the antimicrobial constituents of the A. mexicana extract by TLC-bio autography. Our findings of anticariogenic activity on the *plant* extracts against selected bacteria and phytochemical analysis revealed that these *plants* possess antimicrobial substances.

Keywords: Extraction, Bio autography, Anticariogenic activity, folklore *plants*, secondary metabolites.

1. Introduction

Oral health is integral to general well-being and relates to the quality of life that extends beyond the functions of the craniofacial complex. Dental caries and periodontal diseases are among the most important global oral health problems, although conditions such as oral and pharyngeal cancers and oral tissue lesions are also significant health concerns [2]. According to WHO, dental caries are defined as localized post eruptive pathological process of external origin involving softening of the hard tooth tissue. It is an infectious disease caused by microbe's results in decalcification n and destroying particular infected tooth. It develops by complicated mechanism such as interaction of tooth or saliva with acidogenic bacteria. [3] The pH of a healthy mouth is between 6.2 and 7.0. When the pH is <5.5 the tooth is in acid environment and start demineralization. As the enamel losses its minerals, it starts to breakdown, resulting into formation of cavity [4]. Medicinal *plants* and their products used as a primary source of many drugs from several thousands of years. These *plants* play a significant role in prevention, diagnosis and treatment of various diseases in humans. Herbal *plants* used in medicine are a traditional way to maintain spiritual, physical and mental health. The advantage of using *plants* as medicine is, they are not producing any side effects compared to chemically produced drugs or medicines. *Plants* contains several active ingredients such as phenols, tannins, alkaloids, terpenoids, glycosides etc.4 Folklore *plants* are defined as, the traditional beliefs, customs and stories of a community about medicinal *plants*, passed through the generations by word of mouth. Lactobacillus acidophilus,

Lactobacillus casei, Streptococcus mutants and Staphylococcus aureus were the bacteria used to produce cariogenic effect. As a result some bacterial infections are now essentially untreatable with antibiotics. In a 2003, Institute of medicine report, microbial threat to health, antimicrobial resistance was noted as a paramount microbial threat of the twenty first century, some strain of bacteria are now resistant to essentially available antimicrobial drugs and some remain susceptible to only one. The lack of new drug classes is a consequence of difficulties in discovery of new compounds that has persisted for many years [5]. Now-a-days, dental caries is very common in industrialized as well as under developed and low-income countries. Inferring a history, *plants* are rich in antimicrobial drug source. So, it is priority to discover a herbal treatment for dental caries. The present study was taken up to conduct phytochemical and anticariogenic activity studies of some folklore *plants* viz. seeds of Argemone mexicana, Tender leaf twigs of Pongamia pinnata, fruits of Solanum virginianum, Latex of Thevetia peruviana.

2. Material and Methods

2.1 Collection of *Plant* Material

Argemone Mexicana Seeds, Pongamia pinnata tender twigs, Solanum virginianum Fruits and Thevetia peruviana Latex were collected between June and July were collected from the surrounding fields of Bijapur and Solapur. All *plants* and their products were identified and authenticated by botanist.

2.2 Extraction of *Plant* Material

These elected *plant* materials were shade dried and coarsely powdered. The coarse powder was first extracted by maceration and then by soxhlet extraction method. About 50 gm. of Argemone Mexicana Seeds, Pongamia pinnate tender twigs and Solanum virginianum Fruits powder was subjected to cold maceration with 200 ml of 80% aqueous ethanol for 24 hours [6]. The flask was securely plugged with absorbent cotton and was shaken periodically. The extract was filtered and the marc was air dried. About 50gm of fresh fruits of T. peruviana were collected, washed 2-3 times with distilled water and used for extraction. 50gm of milky white latex was obtained by incising the fruits. The latex was air dried and treated with petroleum ether eliminate any chlorophyll pigments and rubber materials that were present. The air dried latex was then extracted by maceration using 80% aqueous ethanol [7].

2.3 Bacterial Strains for Anticariogenic Activity

A group of bacteria that cause tooth caries were selected. *Lactobacillus acidophilus* (NCIM5306), *Lactobacillus casei* (NCIM5303), *Streptococcus mutans* (MTCC890) and *Staphylococcus aureus* were used in present study to study the antimicrobial activity of the prepared extracts. *L. acidophilus* and *L. casei* were procured from National Collection of Industrial Microorganism (NCIM), CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune-411008, and India. *S. mutans* was procured from Microbial Type Culture and Gene Bank (MTCC), Institute of Microbial Technology, Shanti Path, 39A, Sector 39, Chandigarh, 160036, India. *S. aureus* procured from BLDE Shri B. M. Patil Medical College Hospital & research Centre, Solapur road, Vijayapur 586103.

2.4 Preliminary Phytochemical Screening

Crude extract obtained from extraction process were subjected to phytochemical studies.

Procedure for Detection of alkaloids [8]

Mayer's test: Filtrates of drug extracts are treated with freshly prepared Mayer' reagent. Yellow coloured precipitate shows presence of alkaloids.

Wagner'stest: Filtrates of drugs extract were treated with Wagner' reagent. Formation of reddish-brown precipitate confirms presence of alkaloids

Dragendroff'stest: Filtrates of drugs extract were treated with Dragendroff' reagent. Red precipitate indicates presence of alkaloids.

Hager'stest: Few drops of Hager' reagent were added infiltrates of drugs extracts. Yellow precipitate indicates presence of alkaloids.

Procedure for Detection of Glycosides [9]

Modified Brontrager'stest: 1gm. of drugs extract was added in 5 ml of dilute HCL. 5 ml of ferric chloride was added and boiled on water bath for 10 min. filtrate was treated with carbon tetrachloride and equal amount of ammonia solution. Formation of pink to red color indicates presence of glycosides.

Legal'stest: Drug extract was added in equal amount of water. 0.5 ml of lead acetate solution was added. Filtrate was treated with equal amount of chloroform. 2 ml of pyridine and sodium nitroprusside was added in above mixture. Appearance of pink color confirms presence of glycosides.

Baljettest: Drug extract was treated with sodium picrate solution. Yellow color shows presence of glycosides.

Liebermann Burchard Test: Concentrated Sulphuric acid was added from the side wall of test tube. Appearance of violet ring and blue color indicates presence of glycosides.

Procedure for Detection of Saponins

Froth'stest: 3 gm. of extract was mixed with 10 ml of distilled water. Mixture was shaken vigorously. Honeycomb froth indicates presence of saponins.

Detection of Phytosterols [10]

Liebermann test: Filtrates of drugs were treated with sulphuric acid and acetic anhydride solution.
Blue color shows presence of phytosterols.

Salkowskitest: Extracts of drugs were treated with color of Ormandy few drops of concentrated sulfuric acid. Red color indicates presence of sterols.

Detection of Phenols and Tannins [11]

Ferric Chloride Test: Drugs extracts were mixed with warm water. 2mlof 5% ferric chloride was added. Greenor blue colour indicates presence of phenolic compounds.

Gelatin Test: 1% of gelatin solution containing 10% sodium chloride was treated with drug extract. Formation of precipitation indicates presence of phenolic compound**.**

Lead Acetate Solution Test: Drug extract was treated with 0.5ml of 1% lead acetate solution. Formation of precipitation indicates presence of tannins and phenolic compounds.

Sodium Hydroxide Test: Few drops of sodium hydroxide solution were added infiltrate. Formation of precipitation indicates presence of phenolic compounds.

Shinoda test: Few drops of magnesium ribbon and concentrated hydrochloric acid were treated with extract. Appearance of magenta color indicates presence of phenolic compounds**.**

Detection of Proteins and Amino Acid [12]

Millions Test: 1 ml of Millions reagent was added in 1 ml of filtrate. Mixture was boiled for 1min. after cooling 1% sodium nitrate was added. Development of brick red precipitation indicates presence of proteins.

Biuret Test: 1mloffiltrates of drug extracts was treated with 1ml of Biuret reagent .Development of blue color represents presence of proteins.

Ninhydrin Test: 2% of Ninhydrin solution was prepared by dissolving 2gm of Ninhydrin in 10ml of distilled water. Few drops of ninhydrin solution were added in filtrate. Deep blue color indicates presence of amino acid.

Detection of Carbohydrates [13]

Molisch'stest: 2 drops of alcoholic α-naphthol were treated with filtrate. 2 ml of concentrated Sulphuric acid was added in the tube by side walls. Formation of violet ring indicates presence of carbohydrate.

Benedict'stest: Filtrates were treated with Benedict's reagent and heated on water bath. Formation of orange red precipitate indicates presence of reducing sugar.

Fehling'stest: Filtrates were treated with dilute hydrochloric acid. Fehling's A and B were added. Red precipitation indicates presence of reducing sugar.

Barfoed'stest:1 ml of filtrate was treated 1 ml of Barfoed's reagent. Brick red precipitation shows presence of carbohydrate.

Detectionoffixedoil [14]

Staintest: Small quantity of extract was placed between two filter papers. Oil stain on paper indicates presence of fixed oil.

Soaptest: Few drops of 0.5N alcoholic potassium hydroxide were added to the extract.1drop of phenolphthalein was added in mixture. Mixture was heated on water bath for 2hrs.Formation of soap indicates presence of fixed oil.

2.5 Preparation of Bacterial Inoculum

Broth cultures of the selected bacteria were prepared by inoculating a loopful of bacteria into organism-specific media and incubated at optimal temperature. MRS media was used for *L. acidophilus* and *L. casei*. Enriched infusion heart media for *S. mutans* and Nutrient broth media for *S. aureus* were employed respectively. The bacterial suspension was compared with 0.5 Mc Farmland turbidity standards, which is equivalent to approximately 1×10^8 bacterial cell count per ml. Such prepared bacterial suspensions were used for antimicrobial studies [15].

2.6 Bioassay for Anticariogenic Activity of *Plant* Extracts

2.6.1 Agar well Diffusion Method

The anticariogenic activity of extracts of *Argemonemexicana* (seed), *Pongamiapinnata* (tender leaf), *Solanumvirginianum* (fruit) and *Thevetiaperuviana* (latex) were studied by agar well diffusion method. The *plant* extracts were dissolved in dimethyl sulfoxide and were tested at three concentrations viz. 10, 50 and 100 mg/ml. The agar plates were prepared and labelled for specific bacteria and extract. Afresh bacterial culture of 100 µl having 1×10^8 CFU/ml was spread on agar plates using sterile cotton swab. 6mm diameter well was made With a sterile borer.

The prepared wells were filled with 100 µl of respective *plant* extracts. Plates were placed in there frigate for 30 min. for diffusion of extracts. Then plates were incubated at 37˚C for24 hrs. The zone of inhibition was measured. Tetracycline (100µg/ml) was used as a standard drug for comparison [16]

2.6.2 Determination of Minimum Inhibitory Concentration (MIC) of *Plant* Extracts

MIC was done by broth dilution method. The *plant* extracts were tested at 30, 40, 50, 60, 70 80, 90 and 100 mg/ml. 100µl of bacterial suspension was added to each tube. The tubes were incubated for 24 hrs at 37˚C. 100µl of 0.1% growth indicator 2, 3, 5- triphenyl tetrazolium chloride was added to each tube to find out bacterial growth inhibition. Tubes were incubated for 30 min. under dark condition. Bacterial growth was determined when colorless growth indicator converted into red color [17].

2.6.3 Thin Layer Chromatography

Stationary Phase: Silicagel 60 F254Mobile Phases:

i) Toluene: ethyl acetate (90:10, v/v),
ii) Chloroform: methanol (90: 10,v/v),
iii) Chloroform: ethyl acetate (90:10,v/v)
iv) Chloroform: ethyl acetate: ammonia (90: 9:1, v/v/v). 10µl of *A. Mexicana* seed extract (100mg/ml in ethanol) was spotted on TLC plates and developed with the above mobile phases. After air-drying, the plates were visualized with iodine vapor.

2.6.4 Bio Autography

Direct bio autography was used for the localization of antimicrobial compounds in the seed [18]. After applying seed extract, the TLC plate was developed using chloroform: methanol (90: 10, v/v) and then air dried completely. The plate was placed over sterile nutrient agar and pressed gently. Overnight broth cultures of bacteria viz. *L. acidophilus, L. casei, S. mutansand S. aureus* were made and the turbidity of the suspension was adjusted to that of Mac Farland's standard. When the temperature of the molten nutrient agar (25 ml) was around50^o C, 100 µl of bacterial suspension was added and thoroughly mixed. About 8-10 ml of bacterial suspension was distributed as a thin a layer over TLC plate, which solidified within a few min.

The plate was incubated overnight at 37^o C, sprayed with a 2% w/v aqueous solution of 2,3,5-triphenyl tetrazolium chloride and incubated for further 4 hr. Zone of inhibition appeared as transparent area against reddish background.

3. Result and Discussion

In present study, the anti-cariogenic assay of *plant* material extract formulation against oral pathogenic organism was carried out for the purpose of checking of sensitivity of cariogenic bacteria. *Plant* material were extracted by using 80% aqueous Ethanol and used for further anti cariogenic activity.

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 and presented in Table 1. Glycosides, saponins, fixed oil were absent in *Thevetiaperuviana*. All other phytochemical constituents same like *Argemone mexicana* found in *Solanumvirginianum* and *Thevetiaperuviana*.

Table 1: Phytochemical constituents studies results.

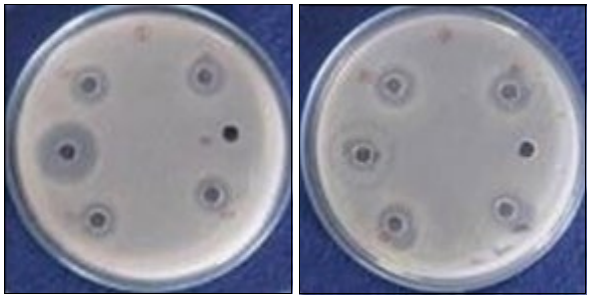
Phytochemical Test	1	2	3	4	5	6	7
Argemonemexicana	+	+	+	-	+	+	+
Pongamipinnata	+	-	+	+	+	+	+
Solanumvirginianum	+	+	+	-	+	+	-
Thevetiaperuviana	+	+	+	-	+	+	+

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

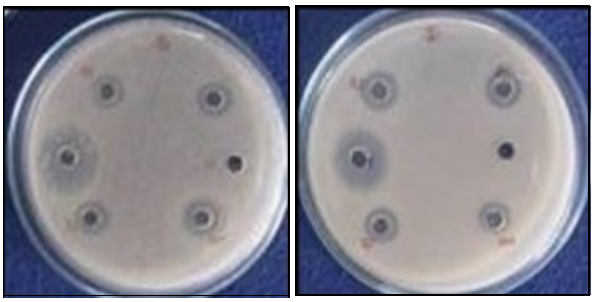
The results of anticariogenic activity of the *plant* extracts and their efficacy werequantitatively assessed by measuring the zone of inhibition. Four *plants* were used for the evaluation of anticariogenic activity. The *plant* extracts were tested for anticariogenic activity by agar well diffusion method. They were tested at three concentrations viz. 10,50and 100 mg/ml. Dimethyl sulfoxide was used to dissolve the *plant* extracts. The result of sensitivity of cariogenic organisms was assessed by recording the presence or absence of Zone of inhibition in diameter. The results are summarized as under Table no.2. At 10 mg/ml concentration, all the *plant* extracts showed no zone of inhibition. The zone of inhibition was observed at 50 and 100 mg/ml concentration of all four ethanolic *plant* extracts. Among the four *plants* extracts, Argemone mexicana showed maximum zone of inhibition compared to other *plants*. Pongamiapinnata and Thevetiaperuviana showed comparatively same zone of inhibition. Solanumvirginianum showed anticariogenic activity but the zone of inhibition was smaller compared to other three *plant* extracts in figure 1.

Table 2: Anticariogenicactivity*plant*extractsat100 mg/ml concentration

Zone of inhibition (mm) at 100 mg/ml concentration					
S. No	***Plant* name**	**L. acidophilus**	**L. casei**	**S. mutans**	**S. aureus**
1.	Argemone mexicana	19	16	17	18
2.	Pongamiapinnata	13.4	14	11	12.5
3.	Solanumvirginianum	7	10.2	9	10
4.	Thevetiaperuviana	13	11.2	13	14
5.	Tetracycline	24.4	27	24.2	26



a) *L. acidophillus b*acteria b) *L. casei*bacteria



c) *S. mutans*bacteria d) *S. aureus*bacteria

Fig 1: Anticariogenic activity of *plant* extracts at 100 mg/ml concentration using

The Minimum Inhibitory Concentration (MIC) values of different formulation of leaves extracts of all the selected *plants* showing highest activity against selected organisms was assessed and summarized in Table no 4. Examining the MIC values of nine samples of various extracts generated the data where the maximum MIC value was found to be all the *plant* extracts showing highest anticariogenic activity against selected bacteria. The *plant* extracts showed significantly remarkable anticariogenic activity against all four bacteria, *L. acidophilus, L. casei, S. mutans* and *S. aureus* with their MIC values ranging from 40 to 90 mg/ml.

Table 3: MIC values of all *plants* ethanolic extracts in mg/ml.

Minimum Inhibitory Concentration (MIC) in mg/ml					
S. No.	***Plant* name**	**L. acidophilus**	**L. casei**	**S. mutans**	**S. aureus**
1.	Argemonemexicana	40	50	50	40
2.	PongamiaPinnata	70	90	60	70
3.	Solanumvirginianum	60	70	80	90
4.	Thevetiaperuviana	70	80	60	80

Argemone mexicana seed extract was found to be the most active extract in theagar well diffusion and MIC studies. Thin layer chromatography of *Argemone mexicana* seed extract done by using 4 different mobile phases viz. toluene: ethyl acetate (90:10, v/v), chloroform: methanol (90:10,v/v), chloroform: ethyl acetate (90:10, v/v) and chloroform: ethyl acetate: ammonia (90: 9:1, v/v/v) were used for TLC studies. After air-drying, the plates were visualized with iodine vapour. The mobile phase toluene: ethyl acetate (90:10, v/v) gave 3 spots. Chloroform: methanol (90: 10, v/v) produced good separation and 5 spots were visible. Separation was not satisfactory with the remaining mobile phases viz. chloroform: ethyl acetate (90: 10, v/v) and chloroform: ethyl acetate: ammonia (90: 9:1, v/v/v) and 2 spots were seen with each mobile phase in figure 3

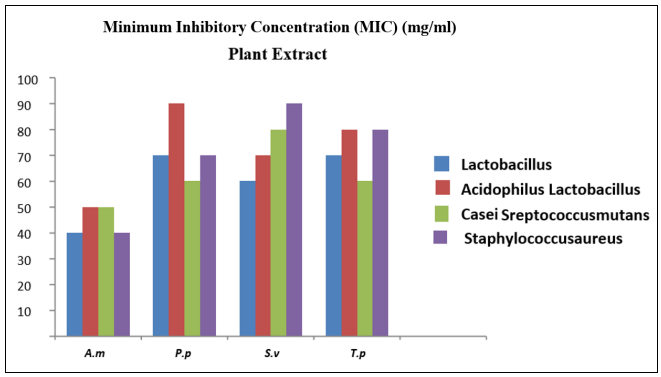
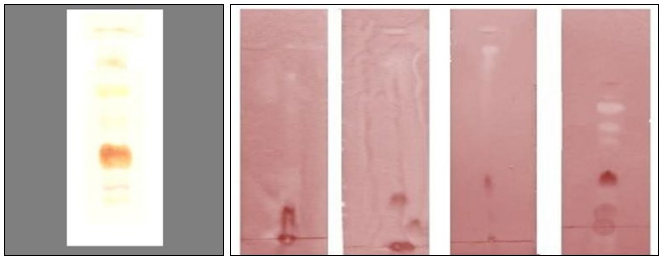


Fig 2: MIC values of *plants* ethanolic extracts in mg/ml

Direct bio autography was performed to localize the antimicrobial compounds on TLC plate. *A. mexicana s*eed extract was applied to the TLC plate and then developed with chloroform: methanol (90: 10, v/v) as it gave good separation. Overnight broth cultures of bacteria viz. *L. acidophilus, L. casei, S. mutans and S. aureus* were prepared. About 8-10 ml of bacterial suspension in molten agar media was distributed as athinalayer over TLC plate, which solidified within afewmin. The plate was incubated overnight at 37° C, sprayed with a 2% w/v aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride and incubated for further 4 hr. Zone of inhibition appeared as transparent area against reddish background.



(A) (B)

Fig 3: Argemonemexicana seed extract (a) TLC, (b) Bio autographic study with L. acidophilus, L. casei, S. mutans, and S. aureus

Out of the 5 spots, spotwithan RF value of 0.25 showed activity with *L. acidophilus.* The spot with an RF value of 0.78 showed activity with *L. casei.* The spot with an Rf value of 0.78 showed activity with *S. mutans.* Out of the 5 spots, spots with Rfvaluesof0.34, 0.46 and0.6 showed activity with *S. aureus* in figure 3.

Conclusion

The extract of different pant material has good sources of as ant cariogenic compound against pathogenic microorganisms, which can be used for treat the infectious diseases. The very good activity of *plant* extracts formulation useful for future development of drug for control of dental carries.

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