WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 12, 670-685. Research Article ISSN 2277–7105

PHYTOCHEMICAL ANALYSIS AND *INVITRO* ANTIMICROBIAL ACTIVITY OF *Alstonia scholaris* R.BR. FLOWERS AGAINST ENDODONTIC PATHOGENS.

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Article Received on
30 April 2018,

Revised on 20 May 2018,
Accepted on 10 June 2018

DOI: 10.20959/wjpr201812-12598

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ABSTRACT

Phytochemical analyses of hexane, ethyl acetate, chloroform and methanol extracts of *Alstonic scholaris* flower was done. All the four solvent extracts revealed the presence of anthocyanins, alkaloids, terpenoids and coumarins whereas hexane, ethylacetate and chloroform extracts revealed the presence of quinines and flavonoids. Phenols are found in only hexane and methanolic extracts. tested against the oral pathogens. Seven microorganisms, responsible for formation of plaque, caries and oral infections, namely *Streptococcus mutans, Enterococcus faecalis, Lactobacillus acidophilus, Staphylococcus aureus,(*Gram-positive bacteria). *Pseudomonas aeruginosa, Escherichia coli (*Gram-negative bacteria) and *Candida albicans* (Fungi) have been selected to assess their susceptibility to various solvent extracts of the flowers. The antimicrobial potential of the extracts was assessed by zone of inhibition method and Resazurin microtiter assay (REMA) plate method. The zones of inhibition obtained for the test organisms ranged between 11- 17mm diameter with the extract concentration of 3mg/ml. These zones indicate that the growth of test organisms was inhibited and they are susceptible to the extracts employed. MIC value of 1mg/ml of the extracts inhibited 28-58% growth of test organisms. The results obtained indicate the antimicrobial potential of flower extracts of *Alstonia scholaris.*

KEYWORDS: *Alstonia scholaris*, Agar well diffusion method, REMA, Bioautography. MIC.

INTRODUCTION

Natural products have contributed much in drug discovery process in the 21st century.[1] They have been the single most productive source of leads for drug development. Most active compounds from traditional medicine sources[2] could serve as good scaffolds for rational drug design. The World Health Organization estimated that approximately 80 percent of the world's population relies primarily on traditional medicines as sources for their primary health care.[3]

The members of the family Apocynacaeae are known for their pharmacological applications as many bioactive molecules[4-6] were reported. *Alstonia scholaris*, a member of Apocynaceae, popularly known as Saptaparni has extensive pharmacological and pharmacognostic value. Extracts obtained from different parts of this plant have been tested for their pharmacological activities like anti-cancer, hepatoprotective, anti-inflammatory, anti-viral and anti-diabetic activities.[7-10] However, reports on the biological activity of flower extracts were scanty.[11-12] The antimicrobial potential of the flower extracts against important oral pathogens has been carried out employing different antimicrobial screening methods.

MATERIALS AND METHODS

Collection of Plant Material

The flowers of *Alstonia scholaris* R. Br. have been selected for extraction of phytoconstituents to screen for their antimicrobial potential against important oral pathogens. This plant belongs to the family Apocynaceae, is an evergreen tree with excurrent canopy. The plant can grow up to a height of hundred meters, with greenish white scented flowers that bloom in umbellate panicles.[13] The species name "*scholaris*" has been derived since the wood was used for making school boards.[14] Picrinine and strictamine are present in flowers.[15] The tree is grown throughout India, Southern China and Queensland as an avenue tree.[16]

Fig. 1: *Alstonia scholaris* flowers (Photograph byRamya.M).

The plant, *Alstonia scholaris* R. Br.has been identified by the Dept. of Botany, Andhra University, Visakhapatnam and the Voucher specimen number 22236 has been deposited in the Botany Department Herbarium(BDH), Andhra University, Visakhapatnam. Flowers were collected by hand picking from healthy and disease free plants from the park of P.M Palem, Visakhapatnam, Andhra Pradesh (India), during the month of October when blooming was at a peak. The collected material was wiped with a clean dry cloth to remove the dust, laid out to dry in shade for 15 days. The dried plant material was further dried in an oven at 70^{O}C for 3 hours, then finely pulverized in an electric grinder. The dry powder was stored at room temperature in airtight virgin plastic containers to prevent contamination.

Physico-chemical parameters

Physico-chemical parameters such as moisture content (loss on drying at 105^{o}C), total ash value, acid insoluble ash value and water soluble ash and swelling index were determined.[17-19] Total ash and acid insoluble ash contents are important indices to determine quality and purity of herbal medicines.

Determination of moisture content (Loss on drying): Preweighed dry flower powder, without preliminary drying, was allowed to dry for 5 hours at a temperature of 105^{o}C, cooled and weighed; it was again dried for 30 minutes cooled and weighed. Then the percentage of moisture content was calculated with respect to the air dried drug.

Determination of total ash value: 2g of accurately weighed flower powder was incinerated in a silica crucible over the burner. The charred material was heated in muffle furnace for six

hours at 600-650^{o}C. Then it was cooled in a desiccator and weighed on the ash less filter paper. Then the percentage of total ash was calculated with respect to air dried drug.

Determination of acid insoluble ash: To the crucible of total ash, 25 ml of dilute HCL added and boiled. Then the solution was filtered through an ashless filter paper and the insoluble matter was collected on the filter paper. It was washed with hot distilled water until the filtrate is neutral. Then the filter paper containing the insoluble matter was dried, transferred to the original crucible and ignited to constant weight. Then the residue was cooled in a desiccator for 30 minutes and weighed without delay. Then the percentage of acid-insoluble ash was calculated with respect to the air-dried drug.

Determination of water soluble Ash: The ash was boiled for 5 minutes with 25ml of distilled water. Insoluble matter collected in crucible or on an ash less filter paper and washed with hot water, ignited and weighed. Percentage of water insoluble ash was calculated with reference to the air dried drug.

Fluorescence analysis of dried flower powder

An important feature of fluorescence is that UV light induces a fluorescent nature in many natural products, Following standard procedure[20], 0.5gms of dried flower powder was taken into clean, dry test tubes. To each tube 5ml of different organic solvents like glacial acetic acid, sulphuric acid, nitric acid, 1Nhydrochloric acid, 5% $FeCl_3$, picric acid, 1N NaOH(aqueous) and 1N NaOH(alcoholic) were added separately. Then, all the tubes were shaken and allowed to stand for about 20-25 min. The solutions were observed under the visible light and UV radiation (254 nm and 366nm wavelength) for their characteristic colour reaction and compared with a standard colour chart and colours were recorded.

Phytochemical analysis of flower extracts

The plant extracts of all solvents were used for the phytochemical analysis for the identification of various classes of chemical compounds using standard protocols.[21-22] alkaloids(Mayer's test), Carbohydrates(Molisch's test), Phenolic compounds and tannins(Ferric Chloride test), Triterpenoids (Liebermann- Burchard test), Flavanoids(Shinodas test), Cardiac Glycosides (Keller Kiliani test), Saponins (appearance of foam upon shaking with water), Acids(Effervescence test) and tests for the presence of Coumarins, Quinones and Anthocyanins.

METHOD OF EXTRACTION

The method used for the extraction is serial exhaustive extraction process[23] (Harborne, 1982) using Soxhlet extractor using solvents of different polarity such as Hexane, Chloroform, Ethylacetate and Methanol respectively at temperatures between 60°C to 70°C[24] (11). 30gms of the dried flower powder was packed in a clean dry cloth and placed in the stem of the Soxhlet apparatus and 150 ml of the solvent was taken in the RB flask. The solvent was allowed to run 4-6 cycles in order to extract efficiently. The extract was distilled by using Steam apparatus until the distillate reaches 10ml quantity then subjected to evaporation in a Rota evaporator under reduced pressure. Each extract was weighed, stored in amber bottles and placed in a desiccator.

TLC analysis of extracts: Pre-coated aluminium silica sheets(MERCK) measuring 5.0 × 2.0cm were spotted with the extract using a capillary tube and allowed to run for 30 - 45 min. The solvent system used was 40% ethylacetate: hexane in the ratio of 1:9. The spraying agent includes 250 ml methanol, 9.5 ml sulphuric acid, 2.7 ml glacial acetic acid and 0.7ml Anisaldehyde. The Retention factor (R_f) values were recorded for each band.

MICRO ORGANISMS USED

The antimicrobial activity of the flower extracts of *Alstonia scholaris* was tested against important oral pathogens[25], *Streptococcus mutans, Staphylococcus aureus, Enterococcus faecalis, Lactobacillus acidophilus (*Gram-positive bacteria). *Pseudomonas aeruginosa, Escherichia coli (*Gram-negative *bacteria)* and *Candida albicans.* All the organisms were cultured from swabs collected from the patients of Department of Endodontics, GITAM Dental Hospital and College, Rushikonda, Visakhapatnam. Organisms were cultured in selective media to produce pure cultures and then maintained at 35°C. Fresh cultures were made prior to conducting antimicrobial tests and cultures in log phase of growth only were used.

Antimicrobial activity of flower extracts of *A. scholaris*

Method 1: Antimicrobial screening activity using Bioautogaraphy

Bioautography[26] is microbial detection method is a hyphenated with planar chromatography technique. It is based on antimicrobial properties of analysed substances. The method used in the present study is direct Bioautography.[25] pre coated aluminium silica sheets(bought from MERCK) were cut into rectangular Tlc plate shape in the dimension of 5× 2.the plates were

spotted with the extract using capillary tube. solvent system used for running TLC 40% ethylacetate: hexane in 1:9 ratio and spraying agent includes 250 ml methanol,9.5 ml sulphuric acid, 2.7 ml glacial acetic acid,0.7ml Anisaldehyde after TLC have been performed on the same TLC plate spray microbial suspension and incubate for 12 hrs at not more than 37°c. then remove the TLC plates, spray freshly prepared Resazurin dye (1gm in 100 ml sterile water) on incubated TLC plates. Re incubate Resazurin sprayed TLC plates for another 3 to 4 hrs. The colour of Resazurin dye is purple it turns to pink colour if viable microbial cells are present. Absence of colour change indicates absence of viable cell

Method 2: Antimicrobial activity using Agar well diffusion method.

Agar well diffusion assay[26] has been employed to determine the antimicrobial potential of the flower extracts of *A. scholaris* by measuring the zones of inhibition obtained with different concentrations of the extracts. The different concentrations of extracts were prepared by dissolving the dried plant extracts in 5% dimethyl sulphoxide (DMSO) to reach a final concentration range of 1.0-3.0 mg/ml. Fresh broth culture of the microorganism was swabbed uniformly on the surface of Mueller-Hinton agar (HiMedia) plates. After 30 min. 3 wells of were made with a sterilized cork borer, different concentrations of 50µl of extracts were dispensed into each well, covered and incubated at 37°C for 16 hours. After incubation, the inhibition zones formed around the wells were measured with HiMedia zona scale. All the assays were carried out in triplicate. A standard antibiotic, Ampicillin (5, 10, 20 µg/50µl concentrations) and control solvent DMSO (5% /50µl) were used as positive and negative controls.

Method 3: REMA (Resazurin Microtiter Assay) plate method to determine Minimum inhibitory concentration (MIC).

Resazurin, an oxidation- reduction indicator, has been used to assess the viability of microorganisms and to determine the minimum concentration of the test sample required to inhibit the growth of microorganisms.[27] Resazurin which is violet in colour oxidizes in the presence of microorganisms to purple colour and indicates viability of microorganisms. Stock concentration of 1% (w/v)Resazurin Dye in distilled water was prepared and stored at -20°C. Dilution of 1:10 ratio was used in assays.

The bacterial cultures were grown in nutrient broth and log phase cultures were used for REMA plate assay.[28] The cultures were diluted with 0.85% normal saline, adjusted to 0.5 McFarland standard at 620 nm with absorbance of 0.65-0.72 (1-2*10^8 cfus/ml). The plant

extracts were serially diluted with DMSO with concentrations of 1.0 mg/ml to 3.0 mg/ml. Pre-sterilized 96 well plates (BIOFIL) were used for REMA plate assay. Into each well 50µl of nutrient broth (HIMEDIA), 20µl of microbial culture and 50 µl of the extract were carefully pipetted. The plates were sealed with aluminium foil and incubated at 37°C for 24 hrs. At the end of incubation period, 30 µl of 0.1 mg/ml Resazurin dye was pipette into each well, incubated for 30 minutes to allow for colour development. Resazurin dye is purple in colour in oxidized state and turns to pink when reduced by viable cells indicating bacterial growth. No colour change indicates the absence of growth of the test organism. The MIC was calculated as the lowest concentration of the extract/compound that inhibited the growth of microorganisms. A change in colour of control well to pink indicated the growth of the bacterial culture and no change in colour of sterile control well indicated absence of contaminants. For comparison, different concentrations of Ampicillin (0.1-0.4mg/ml) were used against the pathogens. All the assays were carried out in triplicate.

Percentage cell inhibition and Total Activity

Percentage cell inhibition at MIC was calculated by reading the absorbance of the contents of that well in I Mark Microplate Reader at 595 nm. Percentage cell inhibition was calculated as follows: Percentage of cell inhibition = [1-(O.D. of treated cells/ O.D. of untreated cells)]*100.

The total activity in ml/gm of plant material was calculated by dividing the quantity extracted from 1 gm of plant material with MIC value. The value indicates the volume to which the extract can be diluted and still inhibits the growth of the bacterial culture.[29]

RESULTS

Extraction efficiency

The amount of extracts obtained and extraction efficacy in different solvents from one gram of plant material (Table 1) shows that there is no substantial difference in the quantity of material extracted by any solvent. However the amount of extract obtained is marginally higher in hexane.

Table 1: Amount of extract (different solvents & Extraction efficiency (%)w/w

Solvent	Hexane	Ethyl Acetate	Chloroform	Methanol
Amount of extract	1.772gm	1.465gm	1.103gm	1.592gm
Solvent	Hexane	Ethylacetate	Chloroform	Methanol

Extraction efficiency(%) 5.90 4.88 3.67 5.301

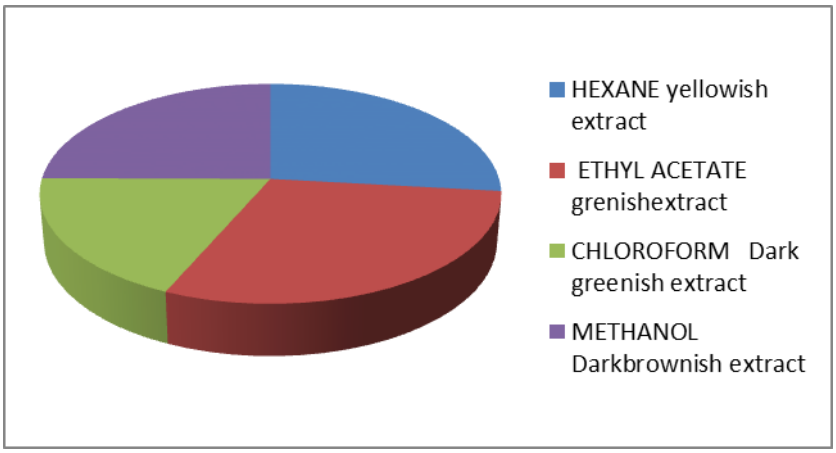

Fig 2: Pie diagram showing extractive values in percentage and colour of extract.

Physico-chemical analysis

The physico-chemical parameters such as ash values of the drug gave an idea of the earthy matter or the inorganic composition and other impurities present along with the drug whereas the moisture content of a drug is important to determine as it prevents microbial growth. Water soluble ash value indicates the presence of cellulosic substances and high acid insoluble ash value indicates the presence of siliceous substances. The values obtained for the flower of *Alstonia scholaris* is shown in Table 2. The moisture content is 84%; the total ash obtained is 8.4%. The water soluble and acid insoluble ash is 1.7% and 2.7% respectively.

Table 2: Physico-chemical parameters of flower powder of *Alstonia scholaris.*

Total Ash	**8.4%**
Water soluble ash	1.7%
Acid insoluble ash	2.7%
Swelling index	No swelling observed
Moisture content	84% initially 19% after drying

Fluorescence analysis of dried flower powder

Fluorescence analysis is also an important tool for the determination of constituents in herbal drugs and it provides an idea about their chemical nature. Fluorescent study of flower powder of ***Alstonia scholaris*** using different chemical reagents showed different colouration under visible light and both short and long UV radiation. The results obtained are shown in Table 3. The dry powder appeared muddy brown in visible light and pale greenish under UV radiation. Among the different solvents tested, flower powder in 1N NaOH(aq.) under long UV radiation did not show any fluorescence whereas, in 1N HCl, 50% Sulphuric acid, 50%

Nitric acid, Acetic acid, FeCl3, picric acid and 1N NaOH(alc) showed characteristic colouration ranging between yellow to light green to green. Some of substances may often be converted into fluorescent derivatives when treated with different chemical reagents though they are not actually fluorescent. This information can be used to assess qualitatively some crude drugs using fluorescence as it is one of the most important parameter of Pharmacognostic evaluation.

Table 3: Fluorescence analysis of the flower powder of *Alstonia scholaris.*

Reagent	UV radiation (366nm wavelength)	UV radiation (254 nm wavelength)	Visible Light
Dry Powder(P)	Light green	Light green	Muddy brown
P+ 1N NaOH(aq)	Brownish	green	Light brown
P+1N NaOH(alc)	Light green	yellow	Yellow
P+ 1N HCl	Green	green	Brown
P+ 50% H_2SO_4	Light green	Light green	Brown
P+ 50%HNO_3	green	green	Brown
P+ Picric acid	green	light green	Brown
P+ CH_3COOH	Light Green	green	Brown
P+ $FeCl_3$	green	green	Yellow

Phytochemical analysis of flower extracts

The results of phytochemical analysis of different solvent extracts of flowers of *Alstonia scholaris* are presented in Table 4. The flowers demonstrated the presence of alkaloids, tritepenoids, carbohydrates, coumarins and anthocyanins in all extracts. Phenolics and tannins are absent in all the extracts with an exception of being present in methanol extract. Cardiac glycosides are absent in all extracts of the flower while quinines are present in all extracts except in methanol. Saponins are present only in choloroform extract whereas flavanoids are present only in hexane extract.

Table 4: Phytochemical Analysis of flower extracts of *A. scholaris.*

Test	Hexane	Ethyl Acetate	Chloroform	Methanol
Alkaloids (Mayer's test)	+	+	+	+
Triterpenoid(Liebermann- Burchard **test)**	+	+	+	+
Coumarins(NaOH test)	+	+	+	+
Tannins(Ferric chloride test)	-	-	-	+
Phenols(Ferric chloride test)	-	-	-	+
Flavanoids(Shinodas test)	+	-	-	-
Saponins(foam test)	-	-	+	-
Carbohydrates(Molisch's test)	+	+	+	+
Quinones	+	+	+	-
Cardiac glycosides (Keller-Kiliani test	-	-	-	-

Acids (Effervescense test)	+	-	-	+
Anthocyanin	+	+	+	+

Antimicrobial activity of flower extracts of *A. scholaris*

Bioautography

Bioautography method of screening showed no growth of microbial culture as it is inhibited by plant extract over TLC plate concluded by no change of colour of Resazurin.

Agar well diffusion Method

Agar well diffusion method showed maximum zone of inhibition 17mm on *E. coli* and minimum zone of inhibition 11mm on *C.albicans.*

Table 5: Zone of inhibition (mm) obtained for selected pathogens with different solvent extracts of of *A. scholaris* flowers.

Organism	Hexane 3mg/ml	Ethylacetate 3mg/ml	Chloroform 3mg/ml	Methanol 3mg/ml	Standard antibiotic 0.02mg/l
E. coli	17	10	14	11	23
P. aeruginosa	14	10	10	12	18
E. faecalis	12	none	12	10	20
L. acidophilus	12	10	13	14	None
S. mutans	12	none	12	12	18
S. aureus	14	10	11	12	30
C. albicans	11	none	12	11	20

***E. coli & P. aeruginosa* (Gram-ve)**

***L. acidophilus, E. faecalis, S. mutans, S. aureus* (Gram +ve)**

Candida albicans (Fungi)

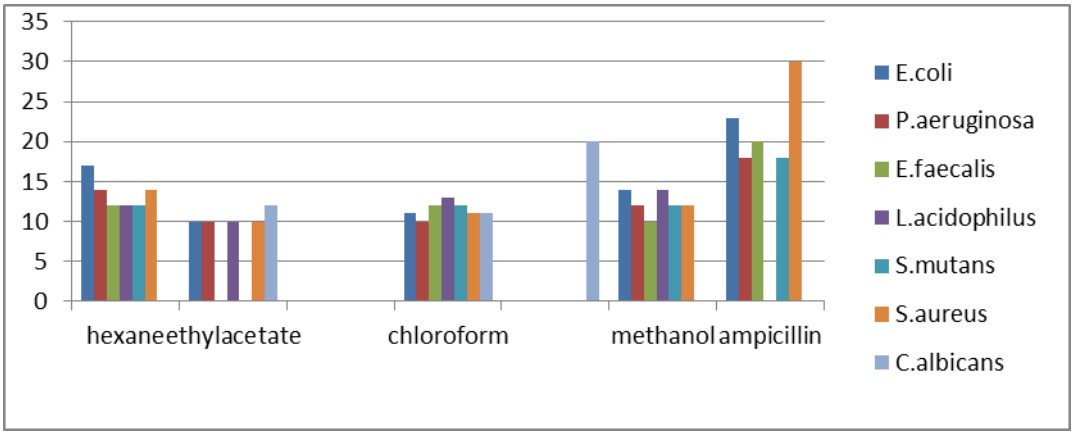

Fig. 3: Zones of inhibition obtained for various oral pathogens using different solvent extracts of *A. scholaris* flowers.

Figure 4: Images showing ELISA READER and 96 well plate used in REMA Assay of anti microbial screening method against *S.mutans*

Table 6: % inhibition of the pathogens by REMA Plate Method.

Organism	Hexane			Ethylacetate			Chloroform			Methanol			Ampicillin		
	Conc(mg/ml)			Conc(mg/ml)			Conc(mg/ml)			Conc(mg/ml)			Conc(mg/ml)		
	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0	1.0	2.0	3.0
	% inhibition			% inhibition			% inhibition			% inhibition			% inhibition		
E.faecalis	*38.4*	*36.0*	*32.0*	*37.8*	*34.0*	*33.2*	*38.5*	*35.2*	*34.0*	*350*	*37.1*	*31.1*	*29.2*	*37.2*	*33.3*
C.albicans	*38.0*	*32.6*	*30.4*	*26.3*	*25.9*	*23.8*	*32.0*	*31.0*	*24.1*	*24.1*	*42.5*	*24.4*	*11.0*	*13.8*	*10.6*
L.acidophilus	*56.9*	*56.3*	*50.6*	*54.3*	*49.2*	*52.1*	*52.0*	*46.7*	*54.5*	*58.1*	*67.6*	*52.6*	*39.4*	*31.5*	*36.0*
P.aeruginosa	*34.0*	*30.4*	*28.0*	*32.7*	*25*	*22.7*	*30.4*	*25.2*	*25.0*	*31.4*	*31.4*	*31.2*	*11.7*	*17.2*	*16.6*
S.mutans	*50.0*	*45.5*	*44.2*	*56.8*	*54*	*46.7*	*63.8*	*50.0*	*64.5*	*48.8*	*49.8*	*42.8*	*80.8*	*83.0*	*83.0*
E.coli	*28.3*	*31.2*	*33.7*	*24.3*	*17.5*	*25*	*25.1*	*20.2*	*31.5*	*31.2*	*35.9*	*31.2*	*19.4*	*20.3*	*27.0*
S.aureus	*37.3*	*36.3*	*34.9*	*34.4*	*31.8*	*29.1*	*34.8*	*29.8*	*30.0*	*30.0*	*31.1*	*21.0*	*3.0*	*12.1*	*12.1*

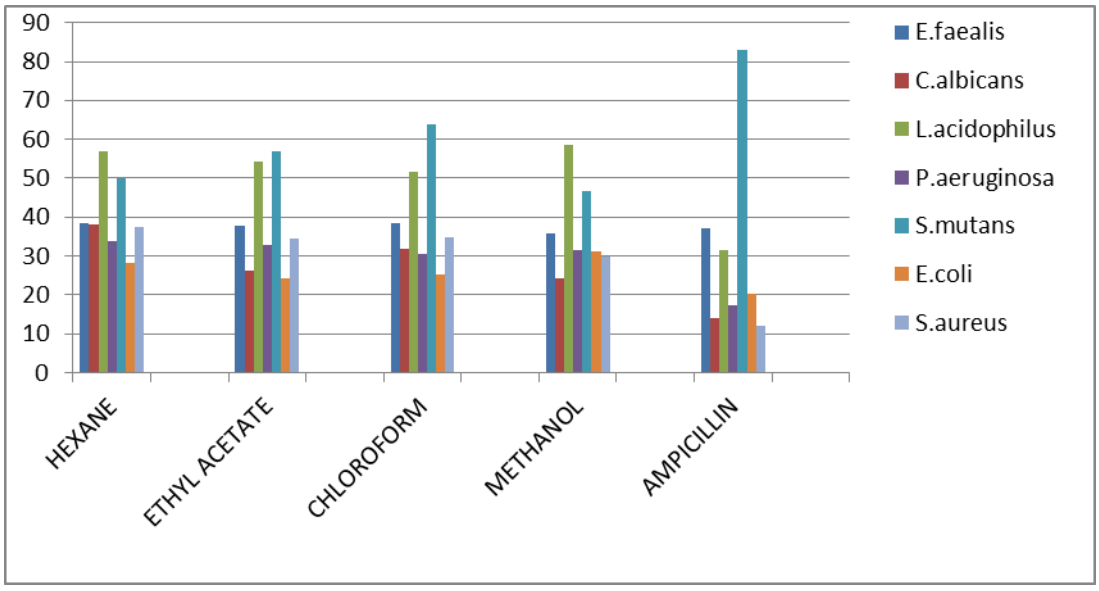

Fig. 5: % inhibition of the oral pathogens at a concentration 50µg/50 µl of different solvent extracts of *A. scholaris* flowers.

Results obtained for the photochemical investigation and antimicrobial screening methods were given in the tables (Nos.4-6). All the seven oral pathogens showed sensitivity in

Bioautography screening studies indicating their susceptibility to these extracts. Of all the extracts tested, hexane extract proved to have broad spectrum antimicrobial potential at 150 µg/50µl (3 mg/ml)concentration with zones of inhibition above 10 mm - 17 mm for all seven oral human pathogens with high susceptibility against gram –ve bacteria (Table 5). Minimum inhibitory concentration was found to be 50µg/50 µl against 10µg/50 µl concentration of standard antibiotic, Ampicillin with a range of 28 – 58% inhibition. The following order of percentage inhibition observed with MIC of 50µg/50 µl of hexane extract for the selected test organisms, ***E.coli* (28.7%) < *P.aureginosa* (33.97 %) < *S.aureus* (37.97%) < *C.albicans* (38%)**, ***E. faecalis* (38.49%)** < ***S. mutans* (50 %) < *L. acidophylus* 5(6.86%)** indicate that ***L. acidophilus*** and ***S. mutans*** the causative organisms in dental plaque formation there by causing dental caries are highly susceptible to the hexane extract. (Table 6) with more than 50% inhibition.

DISCUSSION

Mostly the herbal materials are supplied to the market is shrunken, twisted, rolled and deformed and without trade name and proper identification. So, such drugs can easily be adulterated or substituted. The application of pharmacognostic protocols such as macromorphology, micromorphology, organoleptic tests, ash value, histochemical studies and UV fluorescence study will help in identifying genuine drugs because these tests result in specific results for a particular drug. Identification of the different classes of phytochemical constituents of the plant is an important parameter, which gives an indication of the pharmacological active metabolites present in the plant[30] revealed the presence of alkaloids, flavonoids, coumarins, terpenoids in the stem bark and leaves of *Alstonia scholaris.* The moisture content of the crude is important factor that determine the stability and shelf life of the drug i.e. the lower the moisture content higher will be the stability.[31] Various physicochemical parameters evaluated for the leaf, stem bark and fruit parts as mentioned in WHO guidelines. These parameters are important for detection of drug adulteration or improper handling of raw materials.[32] One such parameter is ash value, which gives an idea of inorganic composition and other impurities in a plant drug. The total ash value is also important for detection of metal, salts, and silica.[33] The difference in the observed activity of various concentration of extract (3mg/ml, 2mg/ml and 1mg/ml) may be due to varying degree of active compounds found in various solvent extracts. The observed maximum % inhibition at 1mg/ml concentration of the extracts than 2 and 3 mg/ml concentrations can be attributed to the presence of antimicrobial compounds with optimum activity in that concentration.

Extracts containing Phenols and Triterpenes were shown to be more efficacious as antimicrobial agents. The presence of Terpenoids, coumarins and alkaloids in thee the solvent extracts supports the antimicrobial potential of the *Alstonia scholaris* flowers in addition to the leaf, bark, stem and other plant parts.[34-36]

CONCLUSION

From the present study it can be concluded that Hexane extract of *A. scholaris* flowers showed promising antibacterial activity against the selected bacteria however the *L. acidophilus* is more susceptible to all the extracts. Antibacterial activity of *A. scholaris* against the test bacteria is a possible indication of newer antibacterial herbal agents in dentistry replacing the conventional synthetic ones. The flowers of this plant can be exploited for isolating promising antimicrobial agents.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the authorities of GITAM University, Visakhapatnam for the facilities provided for carryout this research work. One of the authors (MR) would like to thank the Head of the Department of Pharmacognosy and Phytochemistry, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam for extending all help required for carrying out the current work.

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