IJPSR (2018), Volume 9, Issue 6 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 16 August, 2017; received in revised form, 12 November, 2017; accepted, 17 November, 2017; published 01 June, 2018

PHYTOCHEMICAL SCREENING AND *IN - VITRO* ANTIMICROBIAL ACTIVITY OF *POGOSTEMON QUADRIFOLIUS* (BENTH) OF LAMIACEAE

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Keywords:

Pogostemon quadrifolius, Phytochemicals, Antimicrobial, Antifungal, TLC

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ABSTRACT: *Pogostemon quadrifolius* (Benth.) is a medicinal aromatic shrub in family Lamiaceae which is used as folk medicine in India. This present study deals with the preliminary screening of *Pogostemon quadrifolius* antimicrobial activity and TLC profiling of the crude plant extract. Cold extraction technique was carried out to prepare the crude plant extract, which was further screened for the presence of phytochemicals. The extracts were assessed for antibacterial and antifungal studies by disc diffusion method. Thin layer chromatography was utilized for solvent standardization. Phytochemical analysis revealed the presence of diverse classes of bioactive components. The quantitative analysis of flavanoids, alkaloids and total phenolic content in different extracts varied from each other. Evaluation of antibacterial studies showed that methanolic extract and ethyl acetate extract showed activity against standard and MDR strains respectively. The ethanolic extract also showed fungicidal activity. And finally the solvent standardization was performed for the separation of phyto-constituents by TLC, which showed a very good abundance of active principles (based on their R_f values). All these preliminary reports warrant an in depth analysis on the usefulness of *Pogostemon quadrifolius*as a miracle drug against various ailments.

INTRODUCTION: Plants are being used as remedies for diseases from time immemorial. There is a tremendous increase in the consumption of herbs as an alternate source of medicine to maintain health and improve the quality of life. The chemical components in plants have diverse biological roles and are therefore of therapeutic value. Phytochemicals, the compounds present in plants are valuable source of food and medicine. They are known to have various biological activities such as antimicrobial, antifungal, anti-oxidant activity, *etc* [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(6).2438-45

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(6).2438-45

Phenols, terpenoids, organic acids, lipids, sulphur compounds, amino acids, alkaloids, cytokines, purines, pyridines, sugar and their derivatives and many more are common compounds that are used in phytochemical analysis. Phytochemical analysis helps in determining the medicinal properties in different kinds of herbs. They are naturally occurring in the medicinal plants, leaves, vegetables, roots that have defense mechanism and protect various diseases [2].

Natural products have been the single most productive source used for the development of drugs [3]. Approximately 25 % of drugs prescribed in the United States are plant derived natural products and 74 % of the 119 most important drugs contain ingredients from plants used in traditional medicine. Hence, it can be stated that plants could be a source for the development of new molecules. Pharmaceutical research is now extensively focusing

on natural compounds, for developing active molecules of plant origin [1]. Among the dicot, the member of Lamiaceae contains 45 genera and 574 species with 256 endemic species which is a global distribution. This plant is distributed in India (Khasia, Assam, Sambalpur, Kerala, Godavari District and Garo Hills), Bangladesh (Chittagong) and grown naturally in wastelands. There are about 47 species mainly used for ethanomedicine and traditional medicinal system. It's mainly used for medicinal purpose such as diuretic, sedative, digestive, anti-parasitic, appetizer carminative, anticonvulsant, anti-inflammatory and stimulant [4]. Among the Lamiaceae members the Pogostemon genera have been used by tribal mostly for snake bite. The leaf juice is used as eye drops for hysteria. The plant extract with salt is given for diarrhoea by the Marma in Bandarban.

It is also used to treatment of stomach ache and discomfort [5]. Fever, cough, headaches, wound healing, heart diseases, chicken pox and dys-menorrhea are also treated using this plant. However, there is only limited data on the chemical composition and antimicrobial properties of *Pogestemon quadrifolius* of south Indian origin. This motivated the present investigation on the phytochemical screening of *P. quadrifolius* both qualitatively and quantitatively and focused on establishing the antimicrobial properties of the plant extract.

MATERIALS AND METHODS:

Collection and Identification of Plant Material: The plant samples were collected from collected from uncultivated area of Vazhayoor Hills, Malappuram District, Kerala, South India. The plants samples were preserved as herbarium specimen, identified and authenticated by a taxonomist (CU No: 138487).

Plant Extracts Preparation: The healthy plant leaves were collected and washed thoroughly in distilled water. The leaves allowed drying in shade place for two weeks. Well dried leaf samples were powdered by conventional methods and stored at 4^{o}C until further use. Crude extracts (10% w/v) were made using 5 solvents, *i.e.*, methanol, petroleum ether, chloroform, ethyl acetate and distilled water. The extraction was done by rotary shaker at 10,000 rpm for 24 h. Then each extracts were filtered through fine muslin cloth and the clear filtrate was evaporated to dryness to form the crude extract and stored at 4 ºC for further use.

FIG. 1: *POGOSTEMON QUADRIFOLIUS* HABITAT

Qualitative Screening Test: Qualitative analysis was done to identify the presence of the following phyto-constituents; alkanoids, flavonoids, tannins and phenols, steroids and terpenoids, saponins, carbohydrates, glycosides, proteins and aminoacids using standard procedures.

Alkaloids and Flavonides: About 0.5 g of the extract was stirred separately with a few drops of dilute hydrochloric acid and filtered. The filtrate was carefully tested with alkaloidal reagents such as Mayer's and Dragondroff's. A prominent red and creamy white precipitate in Dragondroff's and Mayer's reagent respectively indicated the presence of alkaloids.

Extracts were treated with few drops of NaOH solution and lead acetate solution. Formation of an intense yellow colour which becomes colourless on addition of few drops of NaOH and formation of yellow coloured precipitate with lead acetate indicates the presence of flavonoids [6].

Terpenoids and Saponins: Extract was treated with trichloro acetic acid. Coloured precipitate indicates the presence of triterpenoids the extract and powder were mixed vigorously with water and stable froth formation indiated the presence of saponins [7].

Steroids and Glycosides: One ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green

fluorescence indicating the presence of steroids 2 ml of extract was treated with glacial acetic acid, one drop 5% ferric chloride and concentrated sulphuric acid. Appearance of reddish brown colour at the junction of the two liquid layers indicates the presence of cardiac glycosides. Extracts were also treated with bromine water and formation of yellow coloured precipitate indicates positive test for glycosides [6].

Tannins and Phenolic Compounds: Extracts were treated with few drops of NaOH solution and lead acetate solution. Formation of an intense yellow colour which becomes colourless on addition of few drops of NaOH and formation of yellow coloured precipitate with lead acetate indicates the presence of flavonoids. Extract was treated with ferric chloride and the production of bluish black precipitate (hydrolysable tannins) and greenish brown colour (condensed tannins) indicated the presence of tannins [8].

Proteins and Amino Acids: Plant extracts were dissolved in one ml of water and treated with Millon's reagent, red colour shows the presence of proteins and free amino acids. Equal volume of 5 % solution of sodium hydroxide and 1 % copper sulphate were treated with the extract.

Appearance of pink or purple colour indicates the presence of proteins and free amino acids. Ninhydrin test was also used to detect the presence of alpha-amino acids and proteins containg free amino groups. The extract was heated with ninhydrin molecules and the formation of characteristic deep blue or yellow colour indicated the presence of amino acids [9].

Carbohydrates: The extracts were dissolved in 5 ml of distilled water and filtered. The filtrate was treated with 2-3 drops of 1 % alcoholic alpha naphthol and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. Violet colour indicated the presence of carbohydrates.

The filtrate was treated with 1 ml of Fehling's A and B and heated in a boiling water bath for 5-10 min. Appearance of reddish orange precipitate shows the presence of carbohydrates. The extract was treated with Benedict's reagent and the appearance of reddish orange colour precipitate indicated the presence of reducing sugar [9].

Quantitative Screening:

Determination of Total Phenolic Content: One ml of the plant extract was evaporated in a petriplate to dryness. Then the dried residue was dissolved in 1 ml of distilled water to get a concentration 1 mg/ml. 100 µl of the dissolved residue was taken and its volume was made up to 3 ml with distilled water and0.5 ml of folin-ciocalteau reagent was added.

Then after 2 min, 20 % of Na_2CO_3 were added and mixed thoroughly. The contents were kept in a boiling water bath for about 1 min. Then the test tubes were cooled in running tap water and the absorbance of the blue coloured complex was taken against blank at 650 nm with the help of UV-VIS Double Beam Spectrophotometer Version 6.51. The total phenol content was calculated and expressed in µg gallic acid equivalence per gram [10].

Determination of Total Flavonoid: Plant crude extract (4 mg) was suspended in methanol (4 ml). 0.25 ml of each suspension was transferred to a tube followed by addition of 1.25 ml distilled water and 5 % $NaNO_3$. After 6 min, 150 µl 10 % aluminium chloride were added and the mixture left for 5 min in the dark. This was followed by adding 0.5 ml 5 % sodium hydroxide and 0.275 ml water and measurement of absorbance by UV-visible spectroscopy at 450 nm wavelength [11].

Determination of Alkaloids: Alkaloids were determines by alkaline precipitation gravimetric method. The measured weight of the sample was dispersed in 10 % acetic acid solution in ethanol to form a ratio of 1:10 (10%). The mixture was allowed to stand for 4h at 28 ^oC. It was later filtered *via* Whattman No. 42 filter paper. The filtrate was concentrated to one quarter of its original volume by evaporation and treated with drop wise addition of conc. aqueous NH_4OH until the alkaloid was precipitated. The alkaloid precipitated was received in a weighed filter paper, washed with 1% ammonia solution dried in the oven at 80^oC. Alkaloid content was calculated and expressed as a percentage of the weight of sample analyzed [12].

Determination of Saponins: Twenty gram of dried leaf powder was mixed with 20 % aqueous ethanol and continuously heated over a water bath for 4 hours. 20 ml diethyl ether was added to the extracts

and shaken. The ether layer was discarded and the purification process was repeated. Sixty ml n-butanol was added and then treated with 10 ml sodium chloride (5% w/v) and heated. The residue remaining after evaporation was weighed and expressed as percentage of leaf powder [13].

Thin Layer Chromatography: The TLC plate were prepared with silica gel-G (activated) which was the stationary phase having a thickness of about 0.5 mm and was activated by incubating at a temperature more than 100 ^{o}C. The test solutions were applied on silica gel-G plate (5x15cm) using a capillary tube. The TLC plate was developed in the saturated chromatographic chamber containing different solvent systems [14].

Test Organisms: One fungal species, *Aspergillus brasiliensis* (ATCC 16404), two gram negative bacteria, *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and one gram positive bacteria *Staphylococcus aureus* (ATCC 25923) was used in the investigation. The MDR strains of the same bacterial cultures *viz*. *E.coli* (CRM-8739) and *Pseudomonas aeruginosa* (CRM-9027) and *Staphylococcus aureus* (CRM-6538) was also used in the study.

Antibacterial Activity: The disc diffusion method was used to test antimicrobial activity against 3 bacterial species. Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances (1mg/1ml) using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms.

Standard antibiotic discs (chloramphenicol) for normal wild strains and for MDR strains (gentamycin, tetracycline, erythromycin, cefotaxin, cefotriaxom, streptomycin 30µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control respectively. The plates were then incubated at 37 ºC for 24 h to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. All the experiments were carried out in triplicates and the standard deviation was recorded [15].

Antifungal Activity: The fungal isolate was allowed to grow on a sabouraud dextrose agar (SDA) (Oxoid) at 25 °C until they sporulated. The fungal spores were harvested after sporulation by pouring a mixture of sterile glycerol and distilled water to the surface of the plate and later scraped the spores with a sterile glass rod. The harvested fungal spores and bacterial isolates were standardized to an OD 600 nm of 0.1 before use. One hundred microliter of the standardized fungal spore suspension was evenly spread on the SDA (oxoid) using a glass spreader. Wells were then bored into agar media using a sterile 6 mm cork borer and the wells filled with the solution of the extract taking care not to allow spillage of the solution to the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 h to allow for proper diffusion of the extract into the media. Plates were incubated at 25°C for 96 h and later observed for zones of inhibition [15].

RESULTS AND DISCUSSION:

Qualitative Analysis of Secondary Metabolites: Medicinal plants are the best sources for chemical ingredients, antimicrobial agents for cure of different diseases. The present investigation, qualitative phytochemical screening test were analysed in leaf extract of *P. quadrifolius*. The result was showed in **Table 1** which indicated the present or absence of compounds of *P. quadrifolius* plant extract. Results showed that, alkaloids and flavanoides was present in high intensity followed by steroids, carbohydrates, tannins, terpenoides, steroids, saponnins, glycosides, proteins and aminoacids. These compounds also can be correlated with the medicinal potential of the plant. Hydrolysable and condensed tannins were both present in the plant extracts. Most natural compounds are derived from primary metabolites such as amino acids and carbohydrates and are generally categorized as secondary metabolites. Secondary metabolites are considered products of primary metabolism but not involved in metabolic activity (alkaloids, phenolics, terpenes, sterols,

flavonoids and tannins, *etc*). The phenolic compound are present in most widely distributed in the plant kingdom [16]. The presence of tannin in plant to protect from animal does not graze [17].

The methanolic extract showed presence of most of the phytochemicals in the preliminary screening study when compared to the other extracts of *Pogostemon quadrifolius.*

TABLE 1: QUALITATIVE ANALYSIS OF *P. QUADRIFOLIUS*

Bioactive components	Methanol extract	Ethyl acetate extract	Chloroform extract	Distilled water extract	Petroleum ether extract
Alkaloides	+	+	+	-	-
Flavanoides	+	+	+	+	+
Terpinoides	+	+	-	+	-
Saponnins	-	+	-	+	+
Steriods	+	+	+	-	-
Tannins	+	-	-	+	-
Phenolic compounds	+	-	+	+	-
Glycosides	+	-	-	+	-
Proteins	+	+	-	-	-
Amino acids					
Carbohydrates	+	+	+	+	-

Qualitative Analysis of Secondary Metabolites: The total alkaloid and saponin content in the plant extract of *P. quadrifolius* is 13.92 % and 10.4 % respectively. The total phenolic content and flavanoid content was found to be more in distilled water and methanol. Ethyl acetate and chloroform did not give any conent of flavanoid in comparison with phenol. The study suggests that the plant contains significantly high amount of alkaloids, saponin and flavanoid and phenol.

TABLE 2: TOTAL FLAVANOID, PHENOL, ALKALOID AND SAPONIN CONTENT IN OF *P. QUADRIFOLIUS*

S. no.	Phytochemical	Distilled water (µg/ml)	Methanol (µg/ml)	Ethyl acetate (µg/ml)	Chloroform (µg/ml)
1	Flavonoid	10 ± 0.63	14 ± 0.52	-	-
2	Phenol	2.5 ± 0.61	15 ± 0.43	20 ± 0.37	23 ± 0.60
			Percentage of yield (%)		
3	Alkaloid		13.92 gm		
4	Saponin		10.4 gm		

TLC Profiling: The separation of phytoconstituents of ethyl acetate and methanolic extracts was performed by TLC using different solvent system and showed a very good abundance of active principles (based on their R_f (Retention factor) values) which is evident from **Fig. 2** and **3**.

TLC profiling of ethyl acetate and methanolic extracts paved the standardization of mobile system for separation of compounds and thus revealing diverse class of compounds with different R_f values (**Table 3** and **4**). Various phytochemicals have different R_f values in different solvent systems. R_f values also reflects the polarity of the compound. Compounds showing high R_f values have low polarity and compound indicating low R_f value goes well with polarity.

It has been reported that TLC profiling of ethanolic extract of plants (*Lantana camara, Pogostemon patchouli, Posmarinus officianalis, Chromolaena odorata*) in different solvent system indicated the presence of different groups of phytochemicals.

Antibacterial Activity: Plant extract of *P. quadrifolius* showed considerably high anti-microbial activity against organisms tested. Measured zone of inhibition obtained against various tested microbes are given in **Tables 5** and **6**. Evaluation of antibacterial studies showed that methanolic extract had more activity that is comparable with the activity of antibiotic discs used as positive control against *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeroginosa* (**Table 5**) but less activity towards MDR strains. While, ethylacetate extract showed more activity against MDR strains (**Table 6**) than other extracts. Mainly, phenolic and flavonoids compounds extracted from the leaves samples shows high antibiotics activity [10]. Further, the phenyl propanoidal derivatives such as phenol and flavonoids have been experimentally proved in

many pharma-cological studies as anti-microbial agents in wide spectrum of bacterial and fungal strains [18]. Among the plant extracts, acetone extract showed maximum inhibition against MDR strains of *Staphylococcus*, *Pseudomonas* and *E. coli* with a zone of inhibition of 4 mm 4 mm and 3.8 mm respectively. Whereas, methanol extract showed maximum inhibition against wild strains of *Staphylococcus*, *Pseudomonas* and *E. coli* with a zone of inhibition of 5.4, 4 and 5 mm respectively. In the present investigation *Pogostemon quadrifolius* plant extracts showed more activity against wild strains than MDR strains.

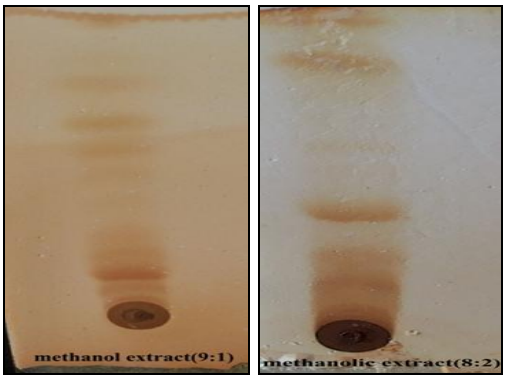

FIG 2: THIN LAYER CHROMATOGRAPHY FOR METHANOLIC EXTRACT 9:1 AND 8:2 RATIO

TABLE 3: RETENTION FACTOR IN TLC USING METHANOLIC EXTRACT

Solvent system used	R_f value
Chloroform:methanol(8:2)	0.6,0.8,0.9
Chloroform:methanol(7:3)	0.82,0.96
Chloroform:methanol(6:4)	0.09,0.5,0.8,0.98,0.9
Hexane:Ethyl acetate(9:1)	0.03,0.12,0.15,0.25,0.64,0.5,0.58,0.84.0.97
Hexane:Ethylacetae(8:2)	0.03,0.13,0.26,0.39,0.46,0.65,0.780
Hexane: Ethylacetate(7:3)	0.19
Hexane:Ethylacetate(6:4)	0.36,0.74
Hexane: Ethylacetate(5:5)	0.8,0.94
D.M:Methanol (9:1)	0.1,0.2,0.42
D.M:Methanol (8:2)	0.09
D.M:Methanol (7:3)	0.6
D.M:Methanol (6:4)	0.6
D.M:Methanol (5:5)	0.8

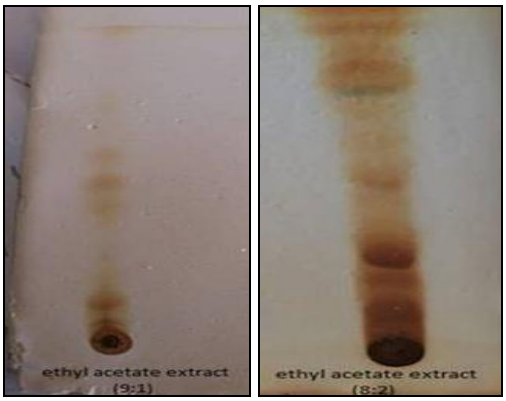

FIG 3: THIN LAYER CHROMATOGRAPHY FOR ETHYL ACETATE EXTRACT 9:1 AND 8:2 RATIO

TABLE 4: RETENTION FACTOR IN TLC USING ETHYL ACETATE EXTRACT

Solvent system used	R_f value
Hexane: ethyl acetate(9:1)	0.007,0.53,0.95
Hexane: ethyl acetate(8:2)	0.12,0.2,0.25,0.28,0.4,0.5,0.625,0.66,0.7,0.75,.0.78,0.8,0.9,0.97
Hexane: ethyl acetate(7:3)	0.16,0.85
Hexane: ethyl acetate(6:4)	0.004,0.24,0.4,0.92
Hexane: ethyl acetate(5:5)	0.13,0.32,0.42,0.81,0.90

TABLE 5: ANTIMICROBIAL ACTIVITY OF *POGOSTEMONQUADRIFOLIUS* ONWILD BACTERIAL STRAINS

Bacteria/Extracts	*E. coli* (mm)	*S. aureus* (mm)	*P. aeuroginosa* (mm)
Methanol	5.0 ± 1.41	5.4 ± 0.66	4.0 ± 0.81
Ethyl acetate	2.3 ± 1.24	2.0 ± 1.00	1.6 ± 0.22
Chloroform	1.4 ± 0.41	1.4 ± 0.56	1.5 ± 0.47
Distilled water	---	---	---

TABLE 6: ANTIMICROBIAL ACTIVITY OF *POGOSTEMONQUADRIFOLIUS* ON MDR BACTERIAL STRAINS

Bacteria/Extracts	*E. coli* MDR (mm)	*S. aureus* MDR (mm)	*P. aeuroginosa* MDR (mm)
Methanol	2.0 ± 1.4	1.3 ± 0.46	2.3 ± 0.94
Ethyl acetate	4.0 ± 0.81	4.0 ± 0.34	3.8 ± 1.0
Chloroform	0.9 ± 0.07	1.2 ± 0.2	1.3 ± 0.22
Distilled water	---	---	---

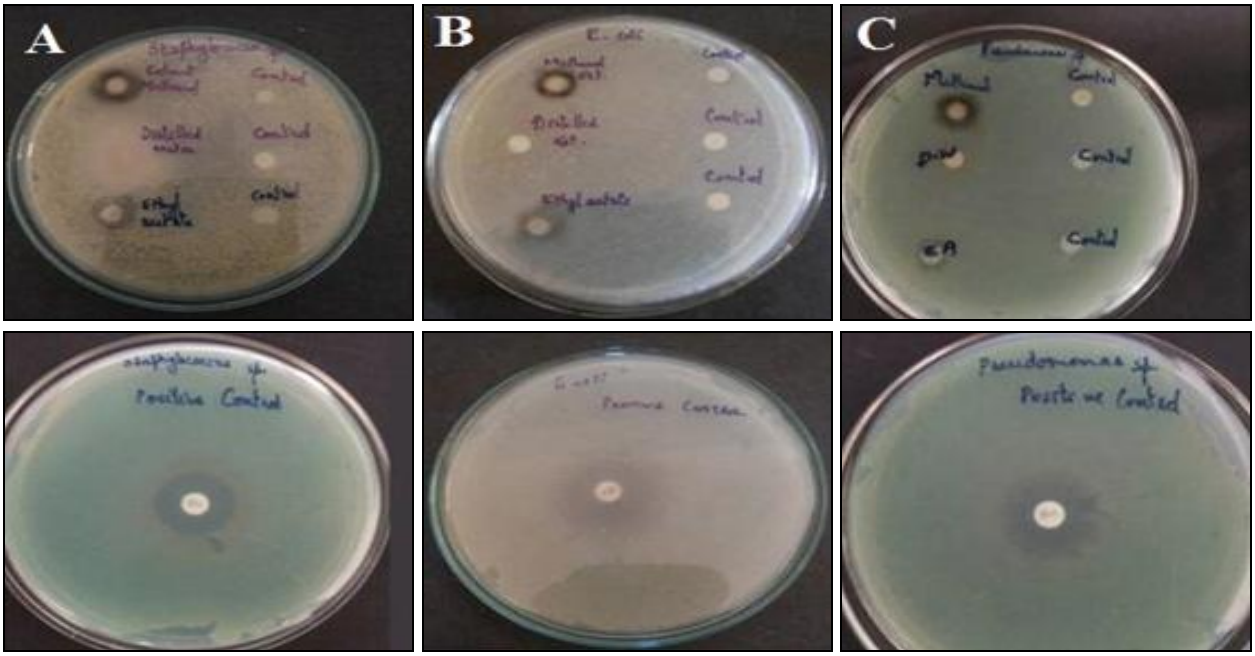

FIG. 4: PLATES SHOWING ZONE OF INHIBITION OF *POGOSTEMON QUADRIFOLIUS* ON WILD STRAINS OF A) *S. AUREUS* B) *E. COLI* C) *P. AEROGINOSA* AND THEIR CONTROL PLATES

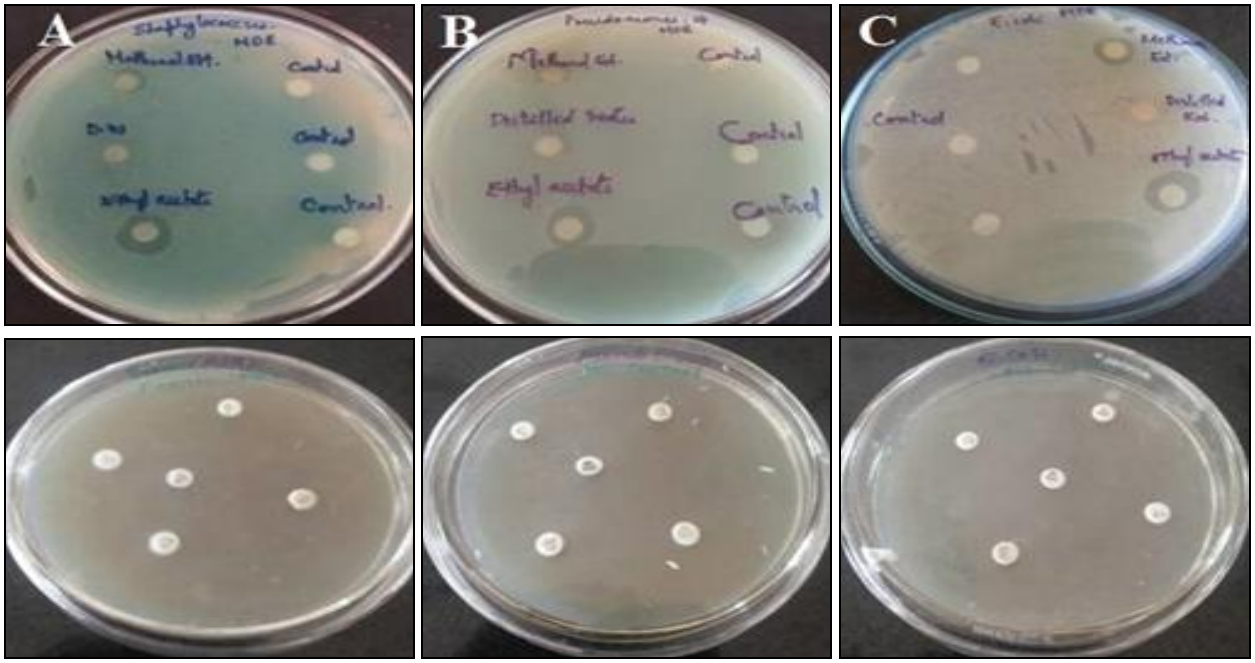

FIG. 5: PLATES SHOWING ZONE OF INHIBITION OF DIFFERENT EXTRACTS OF *P. QUADRIFOLIUS* ON MDR STRAINS OF A) *S. AUREUS* B) *E. COLI* C) *P. AEROGINOSA* AND THEIR CONTROL PLATES

Antifungal Activity: The antifungal activity was screened to determine the fungicidal property of *Pogostemon quadrifolius*. Among the different plant extracts taken for the study, only the ethanolic extract showed antifungal property against *Aspergillus brasiliensis*. **Fig. 6** show zone of inhibitions produced by ethanol extract of *P. quadrifolius* against *Aspergillus brasiliensis* and its control.

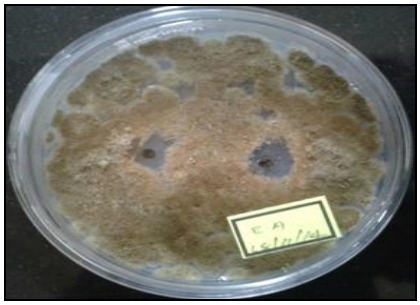

FIG. 6: PLATES SHOWING ZONE OF INHIBITION OF ETHANOL EXTRACT OF *POGOSTEMON QUADRIFOLIUS* ON *ASPERGILLUS BRASILIENSIS*

The largest zone of inhibition was produced by ethanol extract of *Pogostemon quadrifolius* against *Saccharomyces cerevisiae* with a measured zone diameter of 4.5 ± 0.41 mm.

CONCLUSION: The extract of *P. quadrifolius* posses a good source of alkaloids, flavanoids, terpinoides, saponnins, steroids, tannins, phenols, glycosides, proteins, amino acids and carbohydrates. The present findings reveal that different extracts of this plant is a potential source of bioactive components with antibacterial and antifungal properties. Thus there is a future prospective in using the plant extract, into further investigation in the field of pharmacology, phytochemistry and other biological actions for drug discovery.

ACKNOWLEDGEMENT: Nil

CONFLICT OF INTEREST: Nil

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How to cite this article:
Rahman MM, Devi R and Megha PU: Phytochemical screening and *in-vitro* antimicrobial activity of *Pogostemon quadrifolius* (Benth) of Lamiaceae. Int J Pharm Sci Res 2018; 9(6): 2438-45. doi: 10.13040/IJPSR.0975-8232.9(6).2438-45.