IJPSR (2020), Volume 11, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 03 November 2019; received in revised form, 26 December 2019; accepted, 28 December 2019; published 01 January 2020

ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF *HYPTIS SUAVEOLENS*

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Keywords:

Antimicrobial activity, *Hyptis suaveolens*, Polar and non-polar extracts, Phytochemical screening

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ABSTRACT: Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of some known drugs. Components with medicinal properties from plants play an important role in conventional Western medicine. In the ethnopharmacological approach, local knowledge about the potential uses of the plants is very useful as compared to the random approach where indigenous knowledge is not taken into consideration. In the present study, the polar (hydro-alcoholic, aqueous, methanolic and ethanolic) extracts and non-polar extracts (hexane, chloroform and petroleum ether) of whole plant, *Hyptis suaveolens* was screened for antimicrobial activity against drug-resistant strains of *Staphylococcus aureus* and other pathogenic strains (*viz*. *Pseudomonas aeruginosa*, *Bacillus cereus*, *Aspergillus niger* and *Candida albicans*). All the polar extracts of the plant showed significant antimicrobial activity in comparison to non-polar extracts. The studies revealed that the polar extracts are having much significant antimicrobial activity against drug-resistant strains and other pathogenic strains while non-polar extracts are having moderate activity against pathogenic strains while no activity was found against drug-resistant strains. The polar extracts showed potent antibacterial activity against multidrug-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Candida albicans* in the ranges from 5.0 mg/ml to 15.0 mg/ml. The highest antimicrobial fractions of *Hyptis suaveolens* were chromatographed on 2 × 30 cm silica gel 60 open column using a stepwise gradient of methanol and increasing amount of ethyl acetate (20% at each step); ethyl acetate with increasing amount of methanol (10% at each step); and finally at 40% methanol. Collected fractions were evaporated under vacuum and examined by TLC. The antimicrobial fractions were examined using silica gel coated TLC plates to confirm the pure compound by changing the ratios of the solvent system components.

INTRODUCTION: In today's era there is continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections. Green plants represent a reservoir of effective chemotherapeutants and can provide valuable sources of natural pesticides. Reports are available on the use of several plant by-products, which possess antimicrobial properties, on several pathogenic bacteria and fungi [1-4]. *Hyptis suaveolens* is used by various tribal communities of the Chhattisgarh region to cure various diseases like parasitical cutaneous, diseases, infection of the uterus, and as a sudorific in catarrhal condition.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(1).438-44

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(1).438-44

The plant is a stimulant carminative, antispasmodic, antirheumatic, antisuportic bath. It is also used for headache, stomachache and snuff to stop bleeding of the nose. The present study is about the screening of polar and non-polar extracts of the indigenous medicinal plant of Chhattisgarh *viz. Hyptis suaveolens* for antimicrobial activity against drug-resistant and pathogenic strains and qualitative determination of phytochemical constituents.

MATERIALS AND METHODS:

Collection of Plant Material: The plant material (whole plant) of *Hyptis suaveolens* was collected, dried and pulverized to form the powder. The powder plant material was further processed for the preparation of plant extracts by cold percolation method.

Preparation of Plant Extracts: The modified method was adopted for the preparation of plant extracts [5]. Briefly, 20 g portions of the powdered plant material were soaked separately in different solvents for the preparation of polar extracts *viz.* 50% v/v ethanol: distilled water (hydro-alcoholic), distilled water (aqueous), methanol (methanolic) and ethanol (ethanolic) and non-polar extracts (hexane, chloroform and petroleum ether) for 72 h in dark. Each mixture was stirred every 24 h using a sterile glass rod. At the end of extraction, each solvent was passed through Whatman filter paper no. 1 (Whatman, England) The filtrates obtained were concentrated in vacuo using water bath at 30 °C.

Determination of Antimicrobial Activity:

Culture Media: For an antibacterial test, Soyabean Casein Digest agar/broth and Sabouraud's dextrose agar/broth of Hi-Media Pvt. Bombay, India were used for the antifungal test.

Inoculum: The bacterial cultures *viz.* drug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* were inoculated into Soyabean casein digest broth and incubated at 37 °C for 18 h and suspension was checked to provide approximately, 10^5 CFU/ml. The same procedure was done for fungal strains *viz. Aspergillus niger* and *Candida albicans* were inoculated into Sabouraud's dextrose broth separately at 48-72 h.

Determination of Diameter of Zone of Inhibition by Well Diffusion Method: The agar well diffusion method was modified [6]. Soyabean Casein Digest agar medium (SCDM) was used for bacterial cultures. The culture medium was inoculated with the bacteria separately suspended in nutrient broth. Sabouraud's dextrose agar/broth was used for fungal cultures. The culture medium was inoculated with the fungus separately suspended in Sabouraud's dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with plant extracts and solvent blanks. Solvents, 50% v/v ethanol: distilled water (hydro-alcoholic), distilled water, methanol, ethanol, hexane, chloroform, and petroleum ether. Standard antibiotic (Azithromycin, 1 mg/ml) was simultaneously used as a positive control. The plates were then incubated at 37 °C for 18 h. The antibacterial activity was evaluated by measuring the diameter of the zone of inhibition observed. For assaying, antifungal activity of plant extracts, Sabouraud's dextrose agar/ broth medium plates were used.

The same procedure as that for determination of antibacterial property was adopted and then after the diameter of zone of inhibition was observed after 48-72 h. Fluconazole (1mg/ml) was used as the standard for antifungal activity determination. The procedure for assaying antibacterial and antifungal activity was performed in triplicates to confirm the readings of the diameter of the zone of inhibition observed for each of the test organisms.

Determination of Minimum Inhibitory Concentration (MIC): MIC value of potent plant extracts was determined by the method with some modifications [7-8]. The plant extract was prepared in the highest concentration (200 µg/ml) in sterile distilled water and is serially diluted with N-saline (0.85 % NaCl) and a similar quantity of bacterial suspension was added to different test tubes and incubated for 48 h. The inhibition of turbidity appeared in the minimum dose at which the total growth of bacteria gets killed is known as minimum lethal concentration (MLC) while little turbidity appeared in the minimum amount of dose of plant extract which inhibits the growth of bacteria is known as Minimum Inhibitory Concentration (MIC).

Phytochemical Screening of the Extract: Phytochemical screening was performed to test for

alkaloids, saponin, tannins, flavonoids, steroids, sugars and cardiac glycosides [9-10].

Test for Alkaloids: About 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl and was kept in the water bath for about 2 min. 1 ml of the filtrate was treated with Dragendroff's reagent Turbidity or precipitation was taken as an indicator for the presence of alkaloids.

Test for Tannins: About 0.5 g of the sample was dissolved in 10 ml of boiling water and was filtered. A few ml of 6% $FeCl_3$ was added to the filtrate. The deep green color appeared to confirm the presence of Tannin.

Test for Flavonoids: About 0.2 g of the extract was dissolved in methanol and heated for some time. A chip of Magnesium metal was introduced followed by the addition of a few drops of conc. HCl. The appearance of red or orange color was an indicator of the flavonoids.

Test for Saponin: About 0.5 g of the plant extract was stirred with water in the test tube. Frothing persists on warming was taken as evidence for the presence of saponin.

Test for Steroids: Salkowaski method was adopted for the detection of steroids. About 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. To the filtrate, conc. H_2SO_4 was added to form a lower layer. The reddish-brown color was taken as positive for the presence of steroids ring.

Test for Cardiac Glycoside: About 0.5 g of the extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 1% $FeCl_3$. This was underlaid with conc. H_2SO_4. A brown ring obtained at the interphase indicates the presence of deoxy sugar.

A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above the ring and gradually spread throughout this layer.

Test for Reducing Sugars: 1 ml each of Fehling's solutions, I and II were added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2-5 min.

The production of a brick-red precipitate indicated the presence of reducing sugars.

Separation and Purification of Compounds:

Column Chromatography: A funnel was affixed to the top of the column and column exit was closed. The solvent was poured up to 15 cm of the column. Thereafter, the even slurry of silica gel (G) was poured into the column in a single smooth movement, when 4–5 cm bed was formed the column was allowed to flow until the column was packed. The even packing of the column was ensured with trapping of air bubbles inside the packed gel bed. After setting the gel, two-bed volumes of benzene or chloroform were passed throughout the column at an operating flow rate of one drop in one second and the solvent was drained down to the level of gel bed before loading of the sample.

Sample Preparation: Gel filtration is largely independent of sample concentration. The volume of the sample relative to the bed volume is more important for sample preparation. Care has been taken to prepare samples not larger than 1-5% of bed volume. Plant extracts/fractions should be clear and completely dissolved in solvents without particles and any solid contaminants.

Loading of Fraction:

Extracts of Plant Parts: The potent extracts of the plants having the highest antimicrobial activity were subjected to liquid-liquid fractionation. The highest antimicrobial fractions were chromato-graphed on 2 × 30 cm silica gel open column using a stepwise gradient of n-hexane and an increasing amount of ethyl acetate (20% at each step); ethyl acetate with an increasing amount of methanol (10% at each step); and finally at 40 % methanol. Collected fractions were evaporated under vacuum and examined by TLC. Homogeneous fractions were pooled to give a large number of different fractions. These fractions were examined using Silica gel-coated TLC plates to confirm the pure compound by changing the ratios of the solvent system components.

Elution of Compounds: Once the column was filled with eluent, the column was allowed to run. A quick flow rate helps to give good separation. By adjusting the air pressure to give a swift flow rate, different colored bands appeared in the column. After collecting these fractions, the column's progress was monitored by TLC.

Conventional Preparative TLC: Silica gel G used for thin-layer chromatography (TLC) was activated in a hot air oven at 110 °C for one hour. Fractions of the potent plant extracts chromatographed within the column were applied in the form of a band on the TLC plate. The plates used in this method were 0.5-1 mm thick (analytically TLC uses plates of 0.25 mm thickness). This allowed a greater amount of sample to be loaded on the plate. The plates were developed in the solvent, Ethyl acetate: Methanol: Water (EMW) in a ratio of 40:5:4 to separate the polar compounds. Another solvent system used for separation of intermediate polar/acidic compounds was Chloroform: Ethyl acetate: Formic acid (CEF) in the ratio of 5:4:1. For separation of non-polar/basic compounds, Benzene: Ethanol: Ammonium hydroxide (BEA) solvent system was used in the ratio of 90:10:1. A non destructing method was used to detect the compounds. The further developed chromatogram was the use of UV light, to detect all quenching compounds.

Combination of Fractions: From TLC results, fractions were combined according to the similarity of their chemical profile. Combined fractions were placed under air current at a slowly blowing fan to facilitate drying and crystallization. Once dried the fractions were weighed to calculate the total mass fractionated and the crystallized fractions were washed with the combination of solvents to obtain pure compounds. Active fractions were further chromatographed through 3 × 35 cm silica gel column in order to obtain the pure compounds.

Structure Elucidation of Compounds: Identification of compounds was performed by using a combination of different techniques including HPLC and FT-IR. Besides these characterization techniques, R_f values and melting points of the active compounds were also determined.

High-Performance Liquid Chromatography (HPLC): HPLC analysis was performed in NCS Green Earth Pvt. Ltd., Nagpur, Maharashtra, India using a Shimadzu LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzu LC 2010 UV-VIS detector with a thermostated flow cell and a selectable two wavelengths of 190-370 nm or 371-600 nm. The detector signal was recorded on a Shimadzu LC2010 integrator. The column used was a C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. The mobile phase was designed as per the nature of the compound, containing 50% acetonitrile along with 50% Phosphate buffer was used at a flow rate of 3.0 ml/min, column temperature 25 °C. Injection volume was 40 µl and detection was carried out at a specific wavelength having maximum absorbance as calculated by UV absorption spectra at maximum wavelength.

Fourier Transform Infrared (FT-IR) Studies: The IR spectrum of the isolated compound was recorded in NCS Green Earth Pvt. Ltd., Nagpur, Maharashtra, India using a computerized FT-IR spectrometer (Perkin Co., Germany) in the range of 4000-400 cm^{-1} by the KBr pellet technique. The active compound was isolated and identified by spectral analysis using FT-IR comparison with literature values.

RESULTS: The results of the study showed that the polar (hydro-alcoholic, aqueous, methanolic and ethanolic) extracts and non-polar extracts (hexane, chloroform and petroleum ether) of the whole plant, *Hyptis suaveolens* were having antimicrobial activity against drug-resistant strains of *Staphylococcus aureus* and other pathogenic strains (*viz*. *Pseudomonas aeruginosa*, *Bacillus cereus*, *Aspergillus niger* and *Candida albicans*). The results emphasize that the polar extracts of the plant are having much significant antimicrobial activity in comparison to the non-polar extracts.

The study also showed that, amongst the polar extracts, hydro-alcoholic extracts and ethanolic extracts are potent antimicrobial agents in comparison to aqueous and methanolic extracts. Amongst the non-polar extracts, hexane extract is having significant antimicrobial activity in comparison to chloroform and petroleum ether extract. With reference to the strains studied, drug-resistant *Staphylococcus aureus* was found to be sensitive against polar extracts while other pathogenic strains, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Candida albicans* were found to be sensitive against polar and non-polar extracts. The studies revealed that the polar extracts are having much significant antimicrobial activity

against drug resistant strains and other pathogenic strains while non-polar extracts are having moderate activity against pathogenic strains while no activity was found against drug-resistant strains **Table 1; Fig. 1**. The polar extracts showed potent antibacterial activity against multidrug-resistant *Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus niger* and *Candida albicans* in the ranges from 5.0 mg/ml to 15.0 mg/ml **Table 2; Fig. 2**. The phytochemical screening of the polar extracts showed the presence of alkaloids, flavonoids, steroids, saponin and reducing sugars **Table 3.** The potent hydro-alcoholic and ethanolic fraction were chromatographed to obtain the purification of compound with further combination of TLC **Fig. 3**. The single-molecule was isolated and identified as Gallic acid as determined by HPLC and FT-IR techniques **Fig. 4** and **Fig. 5**.

TABLE 1: ANTIMICROBIAL ACTIVITY OF SOLVENT EXTRACTS OF WHOLE PLANT OF *HYPTIS SUAVEOLENS*

Plant extracts (25 mg/ml)/Positive control/Negative control	Diameter of zone of inhibition (mm)			
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Aspergillus niger***	***Candida albicans***
Hydro-alcoholic extract	45.0	28.0	17.0	19.0
Ethanolic extract	40.0	18.0	23.0	27.0
Methanolic extract	32.0	12.0	25.0	27.0
Hexane extract	16.0	5.0	10.0	12.0
Chloroform extract	10.0	2.0	13.0	11.0
Petroleum ether extract	8.0	2.0	7.0	10.0
Azithromycin (1 mg/ml)	35.0	27.0	NT	NT
Flucanazole (1 mg/ml)	NT	NT	25.0	22.0
Hydro-alcohol solvent	NA	NA	NA	NA
Methanol solvent	NA	NA	NA	NA
Ethanol solvent	NA	NA	NA	NA
Hexane solvent	NA	NA	NA	NA
Chloroform solvent	NA	NA	NA	NA
Petroleum ether solvent	NA	NA	NA	NA

*NA, No activity; NT, Not tested

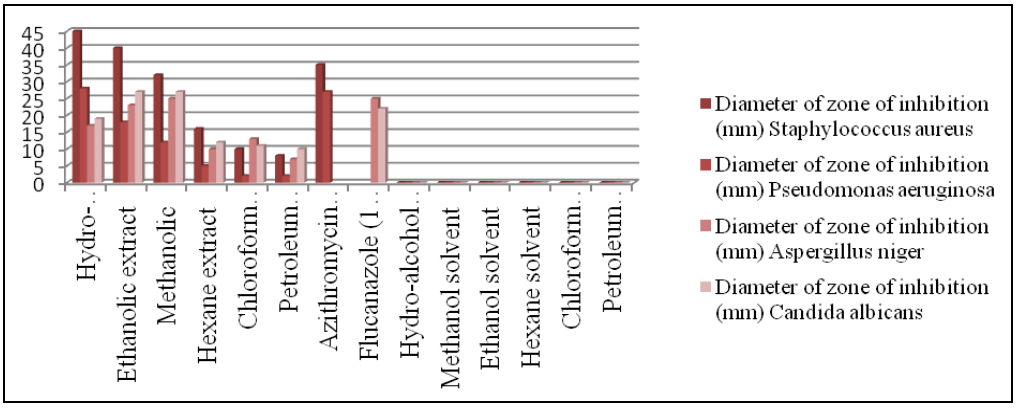

FIG. 1: GRAPHICAL REPRESENTATION OF ANTIMICROBIAL ACTIVITY OF SOLVENT EXTRACTS OF WHOLE PLANT OF *HYPTIS SUAVEOLENS*

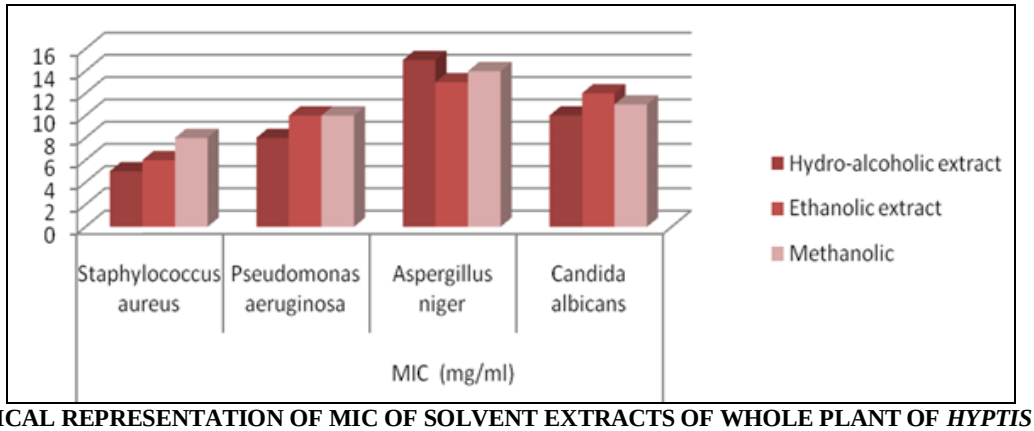

FIG. 2: GRAPHICAL REPRESENTATION OF MIC OF SOLVENT EXTRACTS OF WHOLE PLANT OF *HYPTIS SUAVEOLENS*

TABLE 2: MIC OF SOLVENT EXTRACTS OF WHOLE PLANT OF *HYPTIS SUAVEOLENS*

Solvent extracts	MIC (mg/ml)			
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Aspergillus niger***	***Candida albicans***
Hydro-alcoholic extract	5.0	8.0	15.0	10.0
Ethanolic extract	6.0	10.0	13.0	12.0
Methanolic extract	8.0	10.0	14.0	11.0

TABLE 3: PHYTOCHEMICAL SCREENING OF POLAR EXTRACTS OF WHOLE PLANT OF *HYPTIS SUAVEOLENS*

Solvent extracts	Phytochemical constituents						
	Alkaloids	**Flavanoids**	**Tannins**	**Steroids**	**Saponin**	**Glycosides**	**Reducing sugars**
Hydro-alcoholic extract	+	-	-	+	+	-	+
Ethanolic extract	+	-	-	+	+	-	+
Methanolic extract	+	-	-	+	+	-	+

+, present; -, absent

FIG. 3: TLC OF THE COLUMN FRACTION OF THE SOLVENT EXTRACTS. (1-Hydro-alcoholic fraction; 2- Ethanolic fraction; 3- Aqueous fraction; 4-Hexane fraction; 5- Chloroformic fraction; 6- Petroleum ether fraction)

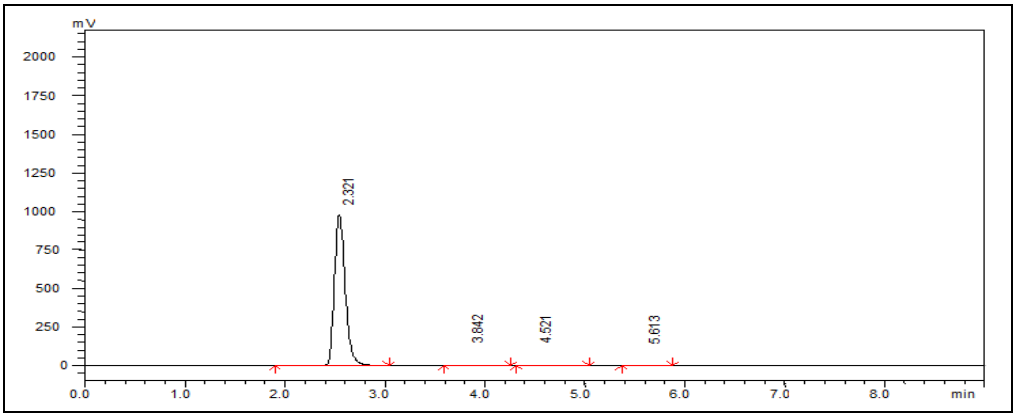

FIG. 4: HPLC CHROMATOGRAM OF HYDRO-ALCOHOLIC FRACTION

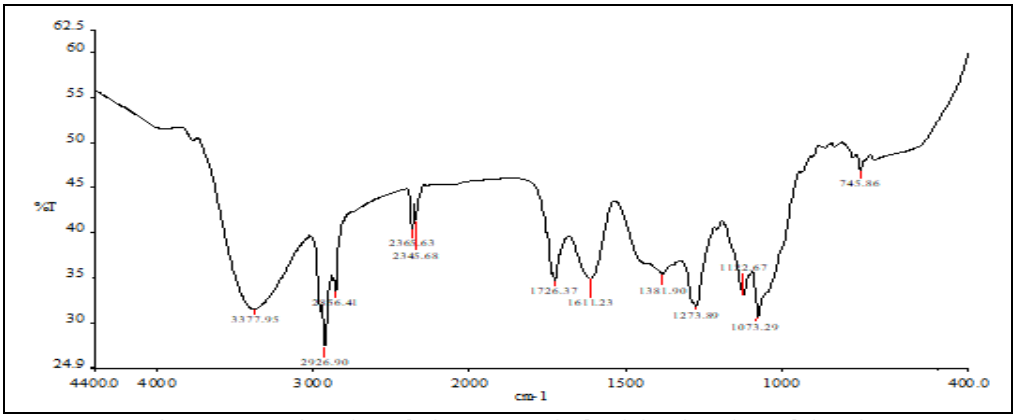

FIG. 5: FT-IR SPECTRA OF HYDRO-ALCOHOLIC FRACTION

DISCUSSION AND CONCLUSION: The significant use of plants as medicine can be traced back over five millennia to ancient documents of early civilizations such as in China, Egypt, India, and the Near East, but is certainly as old as mankind. These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations. Although indigenous knowledge systems rapidly disappear under the influence of western culture, the World Health Organization (WHO) estimates that even today 80% of the world's population exclusively rely on traditional medicine; especially in developing countries' resources to build up primary health care system are still lacking.

In industrialized countries, medicinal plant research had its ups and downs in the last decades. As per the results of the study, polar and non-polar extracts of the whole plant, *Hyptis suaveolens* showed significant antimicrobial activity against drug-resistant strains of *Staphylococcus aureus* and other pathogenic strains, *Pseudomonas aeruginosa, Bacillus cereus, Aspergillus niger* and *Candida albicans*). The results of the study showed that the polar extracts of the plant are much potent in comparison to the non-polar extracts.

The results emphasized that the hydro-alcoholic extracts and ethanolic extracts are potent antimicrobial agents in comparison to aqueous and methanolic extract while amongst non-polar extracts, hexane extract was found to have significant antimicrobial spectrum in comparison to chloroform and petroleum ether extract. The potent hydro-alcoholic and ethanolic fraction were processed via column and TLC to obtain the pure molecule, Gallic acid which showed a single band on the TLC plate and a single peak *via* HPLC. Thus, it can be suggested that the gallic acid isolated from polar extracts of the plant possessed significant antimicrobial activity and thus can be utilized to formulate a drug or can be utilized as a constituent in formulation of drug. The results of the study correlate with the previous findings [11-13].

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: Nil

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How to cite this article:
Sharma K and Dabahadker K: Antimicrobial activity and phytochemical screening of *Hyptis suaveolens*. Int J Pharm Sci & Res 2020; 11(1): 438-44. doi: 10.13040/IJPSR.0975-8232.11(1).438-44.