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Anatomical, phytochemical and *in vitro* antimicrobial studies of *Hyptis suaveolens* L. Poit. of family Lamiaceae

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Abstract: The present study deals with the anatomical, phytochemical and antimicrobial investigations on the Lamiaceae member of *Hyptis suaveolens* (L). Poit, collected from the district Visakhapatnam, Andhra Pradesh, India. *H. suaveolens* L. Poit. the commonest plant in India. It is an undershrub. Stem quadrangular, hairy, leaves aromatic, ovate, tomentose, pale green, turn into the purplish-green at the flowering stage. Anatomical characters observed to be upper and lower epidermis layers of the leaves were covered by multicellular hairs. Vascular bundles were well developed with secondary growth with broad vessels. An attempt has been made to carry out screening phytochemical constituents and antimicrobial ability of various extract of *H. suaveolens* (L.) poit. Shade dried aerial parts were used. The qualitative analysis confirmed the presence of alkaloids, terpenoids, and phenols in leaves. Antimicrobial activity was screened by the agar well diffusion method. Activities of hexane, chloroform and methanol extracts of *H. suaveolens* (L.) poit were tested against *Bacillus subtilis MTCC 441, Enterococcus faecalis MTCC 439, Staphylococcus aureus MTCC 737, Escherichia coli MTCC 443, Proteus vulgaris MTCC 426, Pseudomonas aeruginosa MTCC 1688, Candida albicans MTCC 227, Epidermophyton floccosumMTCC 613,* and *Trichophyton mentagrophytes MTCC 7687*. The antimicrobial activity potential was observed by measuring the width of the inhibitory zones. Hexane and chloroform extract showed mild to moderate inhibitory activity against organisms tested. The methanolic extract showed the highest inhibitory activity against tested microflora. This scientific investigation may be utilized to develop bio fungicide from this plant to control skin diseases in humans.

Keywords: *Hyptis suaveolens*, Antimicrobial activity, Antifungal, Antibacterial, *Epidermophyton flocesum*, *Trichophyton mentagoophytes.*

Introduction

Family – Lamiaceae
Botanical Name – *Hyptis suaveolens* L. Poit.
Vernacular name – Rantulsi
Fls&Frts – October, March

Hyptis suaveolens L. Poit. is an undershrub. Stem quadrangular, hairy. Leaves aromatic, ovate, tomentose, pale green, turn into purplish green. **[Henderson, 1959; Backer and Van de Brink, 1965]**, both leaf surfaces with glandular hairs. Glandular trichomes of aromatic plants containing essential oil **[Simpson, 2006]**. Glandular trichomes are one of the secretory structures found in plants **[Fahn, 1990]** especially in Lamiaceae, Verbenaceae, and Geraniaceae **[Handa, 2008]** families. The scented aroma is caused by essential oils. Many species of the Lamiaceae family with glandular trichomes **[Singh, 2010]** spread over vegetative and floral organs **[Werker, 1993]** and more common in leaves and flowers **[Baran, 2010]**. The morphology, distribution, and a number of glandular trichomes may be as distinguishing characteristics at the subfamily level of Lamiaceae **[Ascensao and Pais, 1995]**. According to Cutler **[Cutler, 1978]**, when a plant with trichomes, it is specific in a form that can characterize the plant species.

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Materials and Methods

Plant material:

Hyptis suaveolens leaves were obtained from Zoo park area of Visakhapatnam, Andhra Pradesh, India. Leaves taken are young, mature and old leaves were observed anatomically by using light microscopes. The anatomical study of the species by Double Staining technique was used (**Microsc, 1953**). Preliminary phytochemical screening of leaves of *H.suaveolens* was studied with reagent screening tests.

Young and matured *H. suaveolens* leaves are collected in the field, and then fixed in formalin, acetic acid, and alcohol solution (FAA). Fresh mature leaves were fixed in F.A.A. 70% alcohol 90cc, glacial acetic acid 5cc, and formalin 5cc. The epidermal peels were prepared by using traditional methods. Epidermal peels were obtained by scraping. The epidermal peels were stained in aqueous safranine mounted in glycerine and studied. Transverse free hand sections of leaf stem were studied with safranine. The botanical illustrations and photos were taken by means of the Olympus binocular optical microscope. The leaf shows dorsiventral differentiation in its internal structure stomata on the abaxial side. The glandular trichomes have unicellular apical cells and a short pedicel has thicker cell wall.

Phytochemical Test:

Preliminary phytochemical screening of *H.suaveolens* leaf methanolic extract is studied for the detection of various chemical constituents. The coarsely powdered plant material is used for extraction with hexane, chloroform, and methanol solvents using Soxhlet extractor. The liquid extract so obtained in each solvent was concentrated by distillation. The condensed solid extract with each solvent was weighted and calculated the percentage in terms of the dry weight of plant materials. The extracts of phytochemical analysis for the identification of bioactive chemical constituents were carried out by using standard reagent tests.

Antimicrobial Studies

Tested organisms:

The antibacterial activity of the crude extracts was tested against Gram-positive Gram-negative bacteria and fungus were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh (INDIA) and the stock cultures were maintained at 4°C until further study. The cultures procured from MTCC were in a dormant state in freeze-dried form.

In vitro antimicrobial assays:

The antimicrobial activity was performed by the agar well diffusion method [**Perez *et al.*, 1990; Murray *et al.*, 1995**] by crude extract of plant species. Which is the most commonly used type for identifying the antimicrobial activity potential.

The antimicrobial activity was carried out by employing 24-hour cultures with given compounds by using the Agar-Well diffusion method. The medium was sterilized by autoclaving at 120° C (15 lb) for about 30 min. About 20 ml of the Nutrient Agar medium used for Bacteria and Potato Dextrose Agar medium for fungal organisms and transferred aseptically into each sterilized petri dish. The plates were left at room temperature in the laminar hood or for solidification. Each plate, a single well of 5 mm diameter was made using a sterile borer. The test compounds were freshly reconstituted with suitable solvents (DMSO) and tested at various concentrations (500, 200 and 100 mg/ml). The samples and the control along with standard (Bacterial/ Ciprofloxacin and Clotrimazole/Fungi) were placed in 5mm diameter well. Petri plates were incubated at 37 ± 2°C for about 12 hrs. Standard (Ciprofloxacin/ Clotrimazole) with 5µg/ml was used as a positive control. The activity diameter of the zone of inhibition was measured using the HiMedia antibiotic zone scale. Triplicate experiments were repeated and observations were listed in Table.

Determination of Minimum Inhibitory Concentration (MIC)

A serial of double dilution affords concentration ranges of 86, 43, 21.5, 10.75, 5.37, 2.68, 1.34 and 0.67 mg/mL. The incubation was carried out during 24 hours at 37° C and turbidity of the medium was examined in each tube looking through at daylight using the human eye

(**Julien *et al.*, 2012**). The transparency of the tubes indicated the antimicrobial effect of the tested extract, while its turbidity shows its ineffectiveness (a sign of bacterial growth). After the MIC, the Minimum Bactericidal concentration (MBC) was determined by a subculture in Mueller Hinton Agar medium of the tubes in which no visible growth was observed. At the same time, control tubes were prepared by culture in agar medium from dilutions of 10°, 10^{-1}, 10^{-2}, $10^{-3}$and 10^{-4} of the starting inoculum corresponding respectively to 100%, 10%, 1%, 0.1% and 0.01% of survival bacteria in culture. The incubation was carried out for 24 hours at 37°C. The MBC was determined in comparison with the control tube with experimental tubes. The first experimental tube in which the number of determined germs is less than or equal to the dilution concentration (10^{-4}) corresponds to the CMB.

Time-Kill Curve

Time kill assay was done in Mueller-Hinton Broth (MHB) medium as described by **Zainin *et al.*, (2013)**. The bacterial inoculums were adjusted to 106 CFU/mL. The AgNPs solution was diluted with MHB media containing bacterial inoculums to obtain the final concentration of 0 × MIC, 0.5 × MIC, 1 × MIC, 2 × MIC, 4 × MIC, and 8 × MIC for each type of bacteria in the total final volume of 1 mL. The cultures were then incubated at 37° C with 150 rpm agitation. The cultures (100 µL) were spread on medium plates at time 0, 0.25, 0.5, 1, 2, and 4 hours. The experiment was carried out in triplicate. The number of colonies on the medium plates was quantified in cfu/mL after incubation at 37° Celsius for 24 hours. For statistical analysis, SPSS statistical package was used to determine the significant ($P < 0.05$) difference among the tested foodborne pathogens.

Results

Morphological Description:

Evergreen, Annual, Erect, herb being approximately 2 meters (120-180 cm) high.

Roots are typically tap root, aerial weak herbaceous.

Stem, woody erect, light green in color, angular young branches cylindrical. Leaves covered by hairs, simple exstipulate, opposite decussate, subsessile unicostate with crenate margin with dimension 8.7-10 x 6.2-7 cm, acute apex triangular petiole, color green. Flower is sub-sessile, bisexual hypogynous, zygomorphic, and blue in color, tetracyclic. Inflorescence a terminal, branched, raceme. Calyx consists of five sepals gamosepalous. Corolla consists of five petals gamopetalous two upper lips become united forming an almost regular, pentamerous. Stamens 4, didynamous, epipetalous and alternate with corolla lobes. Gynoecium bicarperally, syncarpous ovary tetralocular style gynobasic ovary superior. Fruit carcerulus. The placentation: axil.

Fig. 1: *Hyptis suaveolens* (L.) Poit.

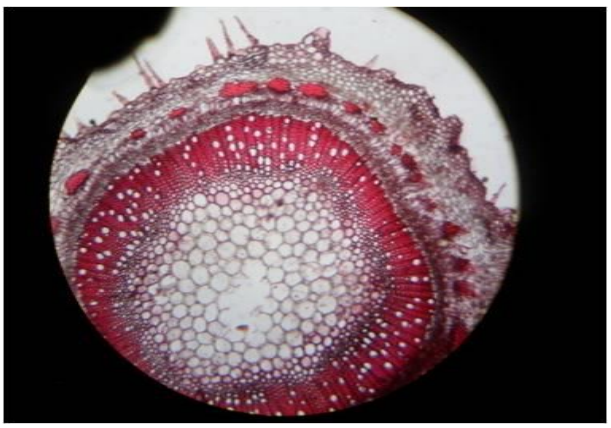

Fig. 2: T.S. Stem of *Hyptis suaveolens*

The anatomical character of Leaf

Both upper and lower epidermises covered by uni-cellular, and multi-cellular glandular hairs. Palisade poorly developed. Mesophyll with compact parenchyma. Midrib and Vascular bundles are well developed having Vessels are broad towards the upper epidermis.

Present studies have shown that organic solvent extracts exhibit higher biological activity than

aqueous extract (**Parekh and Chanda, 2007**). Three solvents, i.e. hexane, chloroform, and methanol, have therefore been used commonly to create the extracts of each plant to identify the best solvent for phytochemical extraction (**Dyana and Kanchana, 2012**). Table 1 shows the preliminary phytochemical analysis. All the preliminary phytochemical screening reports were found to have a large number of phenolics. They were wealthy in phenolics such as flavonoids, and only methanolic extracts show the existence of a minimum amount of tannins. It demonstrates that the species of Lamiaceae are poor in alkaloid structure and present only in methanol extracts of *H. suaveolens* showed the existence of most of the analyzed phytochemicals, i.e. alkaloids, flavonoids, tannins, phenolics, terpenoids, and glycosides. In *H. suaveolens*, glycosides and terpenoids are completely absent as in all other species. Many medicinal plant's bioactive characteristics are due to the existence of various secondary metabolites in them. Secondary metabolites are the primary reason for medicinal plant biological operations such as antioxidant, antidiabetic, hypoglycemic, antimicrobial, anticarcinogenic, anti-inflammatory, anticholinergic, antileprosy, etc. (**Negi, *et al.*, 2011**).

Table 1: Preliminary phytochemical screening of *Hyptis suaveolens*

Plant Name	Solvent	Phenol	Quinone	Tannins	Terpenoid	Saponin
Hyptis suaveolens	Hexane	+	–	+	+	–
	Chloroform	+	–	–	+	–
	Methanol	+	–	+	+	+

Plant Name	Solvent	Alkaloid	Cardiac Glycoside	Coumarin	Flavonoid	Glycoside
Hyptis suaveolens	Hexane	–	–	+	+	+
	Chloroform	–	+	+	–	+
	Methanol	+	+	+	+	+

Antimicrobial activity

The invention and development of antibiotics are the most powerful achievements of modern science and technology for the management of infectious diseases (Nair and Chanda, 2005; Neogi*et al.*, 2008, and Chanda, 2008). In the present study, hexane, chloroform and methanol extracts of *H. sueolences* were studied against seven clinically important microorganisms (Table). All extracts of selected plants were studied against four clinically isolated bacteria *Staphylococcus aureus, Escherichia coli*, *Klebsiella pneumoniae, Proteus vulgaris* and three fungal strains *Trichophyton mentagrophytes, Epidermophyton floccosum,* and *Candida albicans*. Standard antibiotics Ciprofloxacin for bacteria and Clotrimazole for fungi were used as positive controls. From the experimental data, only methanol extracts exhibited moderate to high inhibition zones (activity) against all the microflora screened, Hexane and Chloroform extracts showed only mild to no activity, hence only methanol extracts reports were analyzed. The activity was measured in terms of zone of inhibition in millimeter and classified as Low (<10 mm), significant (10-15 mm) and highest (>15 mm). The antimicrobial activities of different extracts against clinically isolated microbial strains were represented in Tables.

Table 2: List of bacterial organisms

Microorganisms	Type of Strain	MTCC No.	Clinical Significance
Bacillus subtilis	Gram Positive	MTCC 441	Food poisoning
Enterococcus faecalis	Gram Positive	MTCC 439	Urinary tract infections,
Escherichia coli	Gram Negative	MTCC 443	Diarrhoea
Proteus vulgaris	Gram Negative	MTCC 426	Nosocomial infections
Pseudomonas aeruginosa	Gram Negative	MTCC 1688	Urinary tract infections
Staphylococcus aureus	Gram Positive	MTCC 737	Soft tissue infections

Table 3: List of fungal organisms

Microorganisms	Type of Strain	MTCC No.	Clinical Significance
Candida albicans	Fungus	MTCC 227	Urinary yeast infection
Epidermophyton floccosum	Fungus	MTCC 613	Skin and nail infections
Trichophyton mentagrophytes	Fungus	MTCC 7687	Skin Diseases

Table 2: *In vitro* Antibacterial activity of *Hyptis suaveolens*

S.No.	Microorganism	500 mg/ml	250 mg/ml	100 mg/ml	MIC mg/ml
1	*Bacillus subtilis*	16 ± 0.43	15 ± 0.21	11 ± 0.06	75 ± 0.15
2	*Enterococcus faecalis*	13 ± 0.13	10 ± 0.12	08± 0.15	100 ± 0.75
3	*Escherichia coli*	14 ± 0.06	13 ± 0.06	10 ± 0.15	100 ± 0.35
4	*Proteus vulgaris*	14 ± 0.21	12 ± 0.27	11 ± 0.27	75 ± 1.05
5	*Pseudomonas aeruginosa*	21 ± 0.06	17 ± 0.06	15± 0.19	100 ± 1.10
6	*Staphylococcus aureus*	13 ± 0.12	11 ± 0.11	10 ± 0.25	100 ± 0.65

Table 5: *In vitro* Antifungal activity of *Hyptis suaveolens*

S.No.	Microorganism	500 mg/ml	250 mg/ml	100 mg/ml	MIC mg/ml
1	*Aspergillus niger*	23 ± 0.12	18 ± 0.07	16 ± 0.09	100 ± 1.05
2	*Candida albicans*	13 ± 0.23	13 ± 0.09	10 ± 0.23	100 ± 1.05
3	*Epidermophyton floccosum*	18 ± 0.17	14 ± 0.23	12 ± 0.19	75 ± 1.50
4	*Trichophyton mentagrophytes*	20 ± 0.73	16 ± 0.13	11± 0.41	75 ± 0.15

Discussion and Conclusion

The Lamiaceae family have the ability to be great antimicrobial, analgesic, antipyretic, anti-inflammatory, antispasmodic, antioxidant, antidiabetic, antiasthmatic, antidiarrheal, antidote, skin disease antiseptic therapy, arthritic, carminative, toothache, rheumatism, peptic ulcers, hemostatic, anthelmintic, tuberculosis, epilepsy, urinary disease, vaginal discharges, insect biology. Secondary metabolites are characteristics that can be expressed in ecological, taxonomic and biochemical distinction and diversity conditions. The existence of these compounds in the plant's biochemistry is often hard to understand because the plants synthesize them primarily as part of their disease control and herbivore defense mechanism (**Mazid *et al.*, 2011**). Different classes of secondary metabolites are the bioactive compounds that can be used as functional foods in different crops. The therapeutic application of medicinal plants is ascribed to the existence of a broad spectrum of secondary metabolites or phytochemicals such as alkaloids, flavonoids, glycosides, and phenols, all of which are classified as functional foods by different pharmacological operations. Phenols belong to the biggest group of secondary metabolites in crops, primarily in the Lamiaceae family, and display biological multidirectional activity. Flavonoids attract interest as their anti-inflammatory, analgesic, anti-tumor, antimicrobial, antioxidant, and immune stimulant activities are discovered. Monoterpenes are the metabolites commonly found in the anti-inflammatory characteristics of essential oils. Saponins demonstrate multiple pharmacological actions, including anti-inflammatory, antitussive, expectorant, analgesic, and cytotoxic operations. Cardiotonic glycosides are used to treat heart failure (**Vaishali Rai *et al.*, 2013**). Carotenoids are also used to treat retinal disease and glaucoma in addition to being natural coloring agents for food substances and cosmetics. In our research, the methanolic extract of *Hyptis suaveolens* was a rich source of secondary metabolites such as alkaloids, phenols, cardiac glycosides, tannins, saponins, flavonoids, glycosides, terpenoids, coumarins, and quinones. This is in agreement with the previous reports of the several workers (**Subbaiyan *et al.*, 2013; Rajalakshmi *et al.*, 2013; Maithili and Mekala 2015; Shalini**

and Prema Sampathkumar, 2012). As per the literature review that *H. suaveolens* has been mostly studied for its anti-cancer properties. To date, very few studies have been done on the anti-microbial properties of the plant extract. Therefore, this study focuses on the anti-microbial properties of this plant. Despite many published reports dealing with treatment for neurological disorders, little was known about antimicrobial activity of *H. suaveolens* before this study. The outcome of this work supports the validity of the use of the extract of *H. suaveolens* as medicine in ancient medicinal traditions as well as the traditional usage of the studied plants. This extract possesses compounds with antifungal properties that can be used as antifungal agents in new drugs for the therapy against pathogenic dermatophytes microorganisms.

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