How to Cite:
Panigrahi, S., Mishra, U. S., Dixit, P. K., & Mishra, P. R. (2022). Phytochemical screening, isolation and characterization of potential bioconstituents present in hyptis suaveolens. *International Journal of Health Sciences*, *6*(S2), 10767–10778.
https://doi.org/10.53730/ijhs.v6nS2.7995

Phytochemical screening, isolation and characterization of potential bioconstituents present in hyptis suaveolens

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***Abstract*---**The phytoconstituents derived from the indigenous and traditional used plants are the indispensable source for many diseases. In the present scenario especially post Covid 19, many preferred the use of herbal constituent rather than using synesthetic medicines. The study aimed to identify the constituents derived from the leaves of *Hyptis suaveolens* Poit. (Lamiaceae) by separation and isolation of the three main compounds suaveolol, methyl suaveolate and bergatonol, which was achieved by means of repeated column chromatography and repeated preparative thin layer chromatography. Their chemical structures were characterised by IR, 1 H NMR, 13C NMR and LC-MS experiments.

***Keywords*---**hyptis suaveolens, suaveolol, methyl suaveolate, bergatonol.

Introduction

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans as well

Manuscript submitted: 27 March 2022, Manuscript revised: 09 April 2022, Accepted for publication: 18 May 2022

as valuable components of medicines, seasonings, beverages, cosmetics and dyes. Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. (Kundan Singh Bora & Anupam Sharma, 2011)

The concept of evolution of the phytoconstituents from indigenous and traditionally used herbal source is indispensable. Natural products derived from plants are important to cure various disease conditions. Side effects of various allopathic drugs and development of resistance to currently used drugs increased the emphasis on the use of plant materials as a source of medicine for a wide variety of human ailments. Incidentally, plants and herbs are persistently being studied for the discovery of novel therapeutic agents. India is one of the biodiversity centres with the presence of about 20,000 plants having a good medicinal value and used by different traditional communities.

Hyptis suaveolens (L.) Poit. (HS) (Lamiaceae) is a rapid growing perennial herb found in dense clumps along road side and hilly regions. The various phytoconstituents explored are Grassi et al, in 2006 had isolated Suaveolol and methyl suaveolate from the leaves. Essential oils of the leaves contained bioconstituents as pinene, sabinene, p-cymene, terpinen-4-ol sabinene, terpinen-4-ol, terpinolene, 1,8-cineole, pinene, terpinene, caryophyllene and trans-bergamotene (Adeolu *et al.,* 2005; Tchoumbougnang *et al.,* 2005; Ernest & Jose, 1995; Fun *et al.,* 1990). Essential oils as Eucalyptol gama-ellemene, beta-pynene, (+)3- carene, trans-beta-caryophyllene and germacrene (Moreira *et al.,* 2010) was obtained. The whole plant had the constituents as Suaveolic acid, suaveolol, methyl suaveolate, β-sitosterol, β-sitosteryl glucoside, oleanolic acid, ursolic acid rosmarinic acid and methyl rosmarinate (Timsuksai & Suksamran, 2005). Essential oils of the mature flowering twigs, leaves and stems has sabinene, caryophyllene, caryophyllene oxide, abietatriene, terpinen-4-ol, limonene, cis-sabinene hydrate, spathulenol, terpinolene, pinene, p-cymen-8-ol, trans-sabinene hydrate and abietadiene was isolated by Ranaa *et al.,* 2004; Silva *et al.,* 2003. Essential oils of the leaves contain thujene, pinene, camphene, sabinene, pinene, myrcene, phellandrene, terpinene, terpinolene, cimenenol, linalool, fenchol, camphor, 4-borneol, 4-terpinenol, terpineol, eugenol, copaene, elemene, humulene, aromadendrene, cadinene and cadinene (Neucırio *et al.,* 2001; Joseph *et al.,* 2011). Aerial parts contained Hyptadienic acid (Rao *et al.,* 1990), Essential oils of the leaves have limonene; thujane; pinene; phellandrene; 3- cyclohexen-1-ol; 4-methyl-1-(1-methyl ethyl)-3- cyclohexen-1-ol; 3-cyclohexen-1-carboxaldehyde; elemene; octahydro-1,4-dimethylazulene; 5 -8 -H-9 -H-10 -labd-14-ene; 5 -androst-9(11)-en-12-one and 5 -androstan-2,11-dione (Iwu et al, 1990) Aerial parts contained pentacyclic triterpenes (Mukherjee *et al.,* 1984) and the seed coat was composed of L-fucose, D-xylose, D-mannose, D-galactose, D-glucose and 4-O-methyl-D-glucuronic acid (Gowda, 1984). Root 3 -hydroxylup-20(29)-en-27-oic acid, sitosterol -D-glucoside, ursolic acid and botulinic acid (Misra *et al.,* 1983) -Sitosterol, oleanolic acid, urs-12-en-3 -ol-27- oic acid (peltoboykinolic acid) (Misra et al, 1981). Leaves were proved to contain Suaveolic acid and suaveolol (Manchand *et al.,* 1974) Aerial parts stigmast -5-en-3-ol, oleate, and gamma-sitosterol and butyl 11-eicosenoate (Venugopal *et al.,* 2012)

The Leaf and stem (2E)-1-(2-hydroxy phenyl) pent-2-en-1-one (I) and 1-[(3-hydroxy-5, 5-dimethyl cyclohex-3-en-1yl) oxy] hexan-3-one (II) (Jayakumar *et al.,* 2012). Root contained Betulin and betulinic acid according to Sharma *et al.,* 2010. Leaves edible oil 1, 8-Cineole, 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethy, (-)-Caryophyllene oxide and 4, 4, 8- Trimethyl-Tricyclo, Sabinene, β-Caryophyllene, Naphthalene, Torreyol, Phenanthrene, 4-epidehydroabietol, 2-beta, pinene (Hameed *et al.,* 2014); Noudogbessi *et al.,* 2013. Leaf has Allyl Octadecanoate and Octadec-9-Enoic Acid (Mary *et al.,* 2014).

H. suaveolens is traditionally used to treat diabetes mellitus, eczema, fever, cancer, and headache (Nayak *et al.,* 2013 and Jayakumar *et al.,* 2012) The GC-MS analysis of the essential oil from H. suaveolens L. (Poit) was found to produce constituents as cys-ocymene, sabynene ,beta-pynene, 1-octen-3-ol, beta-myrcene, (+)-3-carene, eucaliptol , gama-terpynene, terpinalene, linalool, (+)-fenchole, citronelal, alpha-terpineol, terpineol, linalil propyanate, citronellol, geraniole, beta-bourbonene, beta-ellemene, trans-beta-cariophyllene, germacrene, bicyclogermacrene, gama-ellemene, delta-cedinene , elemol and espatulenol .(Ana C P M, *et al.,* 2010)

It is used traditionally for the treatment of respiratory tract infections, cold, pain, inflammation, fever, skin diseases and diabetes. Leaves of Hyptis suaveolens have been utilized as a stimulant, carminative, sudorific, galactagogue and as a cure for parasitic cutaneous diseases. Crude leaf extract is also used as a relief to colic and stomachache. Leaves and twigs are considered to be antispasmodic and used in antirheumatic and antisuporific baths, antiinflammatory, antifertility agents and also applied as an antiseptic in burns, wounds, and various skin complaints. The decoction of roots is highly valued as appetizer and is reported to contain urosolic acid, a natural HIV-integrase inhibitor. Both leaves and twigs are considered to exhibit antispasmodic activity and are used as source of anti-inflammatory and antifertility agents and also as antiseptic in burns, wounds and various skin complaints [Chatterjee A and Parkash SC, 1995].

The present study is conducted to evaluate the various extracts of H. suaveolens and isolate the compounds from the extracts and further project for characterization of the biomolecules by the use of hyphenated instruments.

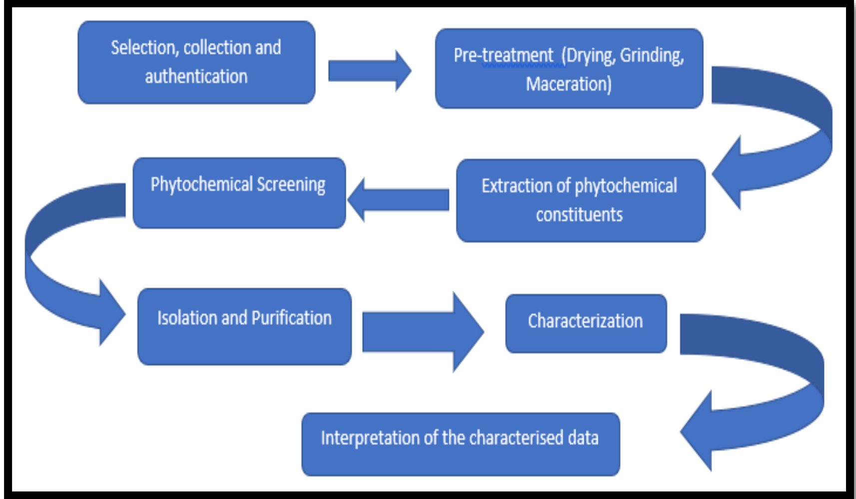


Fig1: Flowchart for the extraction, Isolation, purification and characterization of herbal drugs

Material and Method

Collection and authentication of the plant

The plant material used in this study was leaves of H. suaveolens, collected from road side area of Mohuda district Ganjam, Odisha, India, during spring and was authenticated by the Taxonomist.

Drying and size reduction of the plant

The plant materials were initially rinsed with distilled water and dried on paper towel in laboratory at (37 ± 1°C) for 24 h and milled into coarse powder by a mechanical grinder.

Extraction

The powdered plant material was extracted by Soxhlet apparatus for 72 hours. The extract was prepared using eluent series as petroleum ether, chloroform, ethyl acetate and ethanol. Then the extract was concentrated in vacuum to dryness at 30-40°c temperature, obtaining dried extract. The dried extract was stored and preserved in refrigerator until used for further analysis.

Preliminary Phytochemical Analysis

Tests for carbohydrates and reducing sugars

Molish test: 2 ml of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicate the presence of carbohydrates.

Barford's test: 1 ml of extract and Barford's reagent were mixed in a test tube and heated on water bath for 2 minutes. Red colour due to formation of cupric oxide indicates the presence of monosaccharide.
Fehling's test: To 1 ml of aqueous extract, 1 ml of Fehling's A and 1 ml of Fehling's B solutions were added in a test tube and heated in the water bath for 10 minutes. Formation of red precipitate indicates the presence of reducing sugar.
Benedict's test: Equal volume of Benedict's reagent and extract were mixed in a test tube and heated in the water bath for 5-10 minutes. Solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution which indicated the presence of reducing sugar.
Tests for protein and amino acids: Ninhydrin test: 3 ml of the test solution was heated with 3 drops of 5% Ninhydrin solution in a water bath for 10 minutes. Formation of blue colour indicates the presence of amino acids.

Tests for glycosides

Borntrager's test: To 3 ml of test solution, dilute sulphuric acid was added, boiled for 5 minutes and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and shake it welled. The organic solvent layer was separated and ammonia was added to it. Formation of pink to red colour in ammonical layer indicates presence of anthraquinone glycosides.
Legal's test: 1 ml of test solution was dissolved in pyridine. 1 ml of sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide solution. Formation of pink to blood red colour indicates the presence of Cardiac glycosides.
Keller-killiani test: To 2 ml of test solution, 3 ml of glacial acetic acid and 1 drop of 5% ferric chloride were added in a test tube. Add carefully 0.5 ml of concentrated sulphuric acid by the side of the test tube. Formation of blue colour in the acetic acid layer indicates the presence of Cardiac glycosides.
Tests for alkaloids: To the extract, dilute hydrochloric acid was added, shake it well and filtered. With the filtrate, the following tests were performed.
Mayer's test: To 2-3 ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of white or creamy precipitate indicates the presence of alkaloids.
Hager's test: To 1-2 ml of filtrate, few drops of Hager's reagent were added in a test tube. Formation of yellow colour precipitate indicates the presence of alkaloids.
Wagner's test: To 1-2 ml of filtrate, few drops of Wagner's reagent were added in a test tube. Formation of reddish brown precipitate indicates the presence of alkaloids.

Tests for flavonoids

Lead acetate test: The extract was treated with few drops of lead acetate solution. Formation of yellow precipitate may indicate the presence of flavonoids. Alkaline reagent test: The extract was treated with few drops of sodium hydroxide separately in a test tube. Formation of intense yellow colour, which becomes colour less on addition of few drops of dilute acid, indicate presence of flavonoids.

Shinoda test: To the extract, 5 ml (95%) of ethanol was added. The mixture was treated with few fragments of magnesium turning, followed by drop wise addition of concentrated hydrochloric acid. Formation of pink colour indicate presence of flavonoids.
Test for saponin: Foam test: The extract was diluted with distilled water and shaken in graduated cylinder for 15 minutes. The formation of layer of foam indicates the presence of saponins.

Tests for triterpenoids and steroids

Salkowski's test: The extract was treated with chloroform and filtered. The filtrate was added with few drops of concentrated sulphuric acid, shaken and allowed to stand. If the lower layers turn red, sterol are present. Presence of golden yellow layer at bottom indicates the presence of triterpenes.

Libermann-burchard's test: The extract was treated with chloroform. To this solution few drops of acetic anhydride were added, boiled and cooled. Concentrated sulphuric acid was added through the sides of the test tube. Formation of brown ring at the junction of two layers, if upper layer turned green, indicate presence of steroids and formation of deep red colour indicate presence of triterpenoids.

Tests for tannin and phenolic compounds

Ferric chloride test: Some amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added. Formation of blue, green or violet colour indicates presence of phenolic compounds.
Lead acetate test: Some amount of extract was dissolved in distilled water. To this solution few drops of lead acetate solution was added. Formation of white precipitate indicates presence of phenolic compounds.
Dilute iodine solution test: To 2-3 ml of extract, few drops of dilute iodine solution were added. Formation of transient red colour indicates presence of phenolic compounds.

Isolation of Phytoconstituents

The chloroform, ethyl acetate, and ethanol extracts of *Hyptis suaveolens* were subjected for column chromatography after qualitative phytochemical screening

Thin layer Chromatography (TLC)

Chromatography is widely used for the separation, isolation, identification, and quantification of components in a mixture. Components of the mixture are carried through the stationary phase by the flow of a mobile phase (Wagner, and Bladt, 1996; Stahl, 1969). Separations are based on differences in migration rates among the sample components. TLC is chosen over other chromatography methods because it is a simple, quick and inexpensive procedure and very sensitive to even microgram amount of sample mixtures (Skoog, 2004). TLC is a mode of liquid chromatography in which the sample is applied as a small spot or streak to the origin of a thin sorbent layer such as silica gel, alumina, cellulose

powder, polyamides, ion exchangers or chemically bonded silica gel supported on a glass, plastic, or metal plate. This layer consists of finely divided particles and constitutes the stationary phase. The eluent or mobile phase is a solvent or a mixture of organic and/or aqueous solvents in which the spotted plate is placed. The mobile phase moves through the stationary phase by capillary action, sometimes assisted by gravity or pressure. The effectiveness of the separation depends on the mixture to be separated, the choice of the mobile phase and the adsorption layer and the term retention factor Rf, is commonly used to describe the chromatographic behaviour of sample solutes. The Rf value for each substance is the distance it has moved divided by the distance the solvent front has moved. The solvents, during this research for each crude extract, were chosen by trial and error. The selection was made on the basis of best resolution.

Preparation of plates

Slurry of silica gel G and distilled water was prepared in a pestle with continuous triturating with mortar. The slurry was spread evenly on clean grease free glass plates. The plates were dried in air and thereafter heated in oven at 110 0C for about 30 minutes to activate them (Koehn, 2005).

Preparation of samples

Approximately 10 mg of material was dissolved in respective solvents and was used for spotting on TLC plates.

Application of samples on TLC plates

Samples were applied on the TLC plates with the help of a capillary tube at a distance of about 0.5 cm from the developing solution. The solvent from the plate was removed by air drying and position of the spot was marked.

Saturation of TLC chamber

The inner wall of the chamber was lined with filter paper on three sides, the solvent system was poured up to a height of about 1 cm from the base, grease was applied on the rim of the chamber and it was covered with a glass plate. The chamber was allowed to stand for about 30 minutes and by that time the filter paper inside the chamber was completely drenched by the solvent system, making the chamber completely and evenly saturated with solvent system.

Development of TLC plates

The plates were placed vertically into a solvent vapor saturated TLC chamber and allowed to develop till the mobile phase had moved about 80% from the spotting line; the plate was removed from the developing chamber and dried.

Detection of TLC plates:

The eluted spots, representing various fractions/compounds, were visualized by different detection methods.

i. The plate was visualized at UV–254 nm and UV-365 nm.
ii. The plate were exposed to iodine vapor and observed.

The TLC profile was examined to determine variation in band size and colour intensity of chloroform

Characterization of Phytoconstituents
Characterization is done by using, NMR and LC-MS

Result and Discussion

The preliminary qualitative analysis of various extracts showed the presence of alkaloids, steroids, glycosides, saponins, flavonoids, and tannins and phenolic compounds shown in (Table 1).Phytochemical screening of the prepared extracts was conducted with various qualitative tests to identify the presence of chemical constituents. To perform the tests, the following chemicals and reagents were used: steroids with chloroform and sulphuric acid, tannins with ferric chloride solution, gum with Molish reagents and concentrated sulfuric acid, flavonoids with Mg and HCl and saponins with the capability of producing suds. Alkaloids were tested with Mayer's reagent, Hager's reagent and Dragendorff's reagent. These were identified by characteristic color changes using standard procedures (Ghani, 2003).

Table 1: Qualitative phytochemical analysis of Hyptis suaveolens leaves

Chemical constituents	Chloroform extract	Ethyl acetate extract	Ethanol extract
Carbohydrates	-	-	-
Proteins	-	-	-
Amino acids	-	-	-
Flavonoids	- +	- +	+ +
Tannins & phenolic compounds	-+	+ +	+ +
Glycosides	-	-	- +
Saponins	-+	-+	+ +
Steroids	-+	+ +	+ +
Alkaloids	-+	+ +	+ +

Absent. - - +: Weakly present. + +: Strongly present

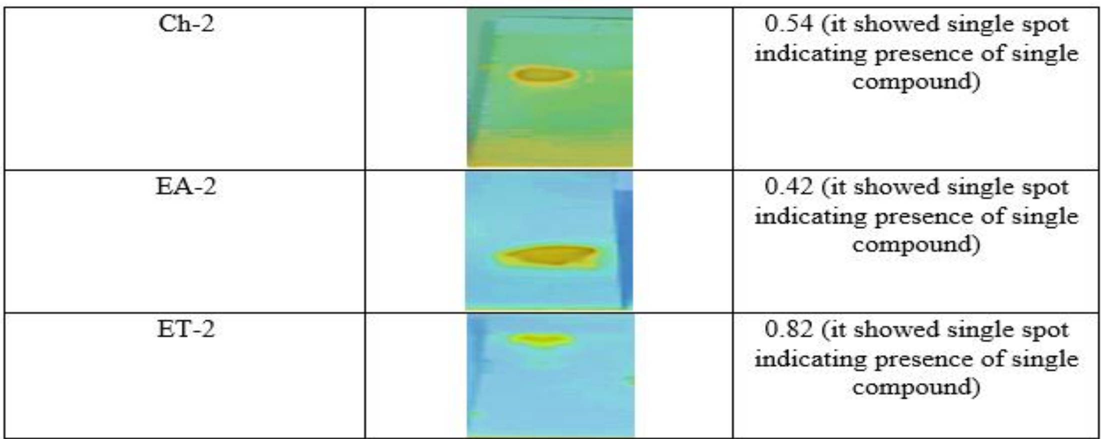
The column chromatography was performed. The different fractions were obtained after successful dripping of solvent for up to 78 hrs. The fractions which possess single colorations were proceeded for further analysis. More precisely single-coloured fractions were selected as it indicates presence of only one type chemical compound. The fractions obtained from each plant extract were named as given in following table

Table 2 - Column Fraction

Plant Name	Extract		
	Chloroform	Ethyl acetate	Ethanol
Hyptis suaveolens	Ch-2	EA-2	ET-2

The above fractions were subjected for TLC, to confirm whether it contains single compounds or not. The TLC analysis of extract which was eluted using the mobile phase (n-hexane: ethyl acetate) (7: 3) showed after keeping it in the Iodine chamber. The obtained TLCs with Rf values are tabulated in table given below in Table 3

Table 3: TLC profile of *Hyptis suaveolens* leaves



Ch-2		0.54 (it showed single spot indicating presence of single compound)
EA-2		0.42 (it showed single spot indicating presence of single compound)
ET-2		0.82 (it showed single spot indicating presence of single compound)

From the TLC analysis all the fractions displayed single spots with the strong indication of presence of single chemical compound. These fractions were subjected for GC-HRMS and NMR analysis to identify the present chemical compound.

Names of Identified Chemical Constituents from each extract

After qualitative and quantitative phytochemical screening, the following compounds have been identified which were later confirmed by spectral analysis From the spectral data the compounds confirmed are given in the following table:

Rutin (Ch-2)	Suaveolic acid (EA-2)	Bergamotol (ET-2)
OH, OH, HO, O, O, OH, O, OH, O, OH, H_3C, O, O, OH, HO, OH, OH	CH_3, CH_3, CH_3, OH, HO, CH_3, O	H_3C, HO, CH_3, CH_3

In the present investigation, we have isolated the rutin, Suaveolic acid and Bergamotol was found in Hyptis saveolence. Rutin found is a bioflavone, which has quercetin-3-O-rutinoside and sophorin, is the glycoside combining the flavanol quercetin and the disaccharide rutinose. Suaveolic acid is a terpenoid are widely distributed throughout the plant and known to have many biological effects. Bergamotol was also found as a constituent having a moiety as oleanolic acid. From this phytochemical screening, chromatographic analysis, and spectral evidence it was confirmed as the compound Ch-2 as Rutin, EA-2 as Suaveolic acid and ET-2 as Bergamotol. The constituents have long been attributed to many pharmacological activities.

Conclusion

Phytochemical investigation showed the presence of imperative phytochemicals such as flavonoids, triterpenoid, and steroids. TLC analysis of leaves provided standard fingerprints for the identification and isolation of triterpenes. This phytochemical evaluation afforded three compounds on column chromatography and repeated crystallizations.

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