



Research Article

Comparative Phytochemical, antimicrobial and antioxidant studies on two Lamiaceae *Ocimum* species of *O. tenuiflorum* L. and *O. tenuifolium* L. from Visakhapatnam, Andhra Pradesh, India.

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Abstract: *Ocimum tenuiflorum* and *Ocimum tenuifolium* are the most popular traditional plants in the history of India. Present investigation is to compare the phytochemical, antimicrobial and antioxidant parameters of organic solvent extracts of hexane, chloroform and methanol. Qualitative phytochemical tests were used to detect the presence of terpenoids, flavonoids, tannins, saponins, glycosides and phenols, while two quantitative methods; Ferric Reducing Antioxidant Power (FRAP) and diphenyl -1, 2-picryl hydrazyl (DPPH) were used to determine the antioxidant potential. The antimicrobial activity was determined by Agar well diffusion method and was screened against seven micro organisms. The highest antimicrobial, antioxidant activities were observed in the methanol extracts of *O. tenuiflorum* when compared with *O. tenuifolium*. Plant species. While the hexane extract showed the least activity irrespective of the method used. The presence of aromatic active phytochemical substances with antioxidant activities may provide substantial basis for the use of these plants in ethnomedicine.

Keywords: Phytochemical analysis; Anti-microbial activity; *Ocimum tenuiflorum*; *Ocimum tenuifolium*

Introduction

Ocimum tenuiflorum (Krishna Tulasi) formerly called *Ocimum sanctum* and *O. tenuifolium* (Rama Tulasi) plant species belongs to the family Lamiaceae class Dicotyledons and it is the largest family of the order Lamiales. Lamiaceae a family of flowering plants commonly known as the mint family. These are mostly herbs/shrubs comprising about 200 genera and 7,000 species. Indian basil is an important symbol in the Hindu religious tradition. Tulsi, or Holy Basil has been described as the “Queen of plants” and the “mother medicine of nature” due to its perceived medicinal qualities (Singh *et al.*, 2010). It has been one of the most valued and holistic herb used over years in traditional medicine in India. Almost every part of the plant has been found to possess therapeutic properties. Aromatic herbs, aerial parts with glandular hairs, quadrangular stems, and tyrsus inflorescences. Lamiaceae is distributed nearly worldwide, and many species are cultivated for their fragrant leaves and with zygomorphic flowers. The family is particularly important to humans useful for flavour, fragrance and for medicinal purposes.

In India Tulsi plant is known as Queen of herbs, worshiped equivalent to God as it has its name mentioned in our holy scriptures for worshiping Lord Vishnu and his avatars like Krishna and Ram (Rahman *et al.*, 2011). Tulsi have been used in medical practice for thousands of years and have made a great contribution to maintain human health. The main bioactive components in medicinal plants are considered to be constituents of essential oils (Singh *et al.*, 2010; Wu *et al.*, 2016). There are many advantages and benefits associated with the use of these medicinal plants, the main ones being their cost-effectiveness and global availability. Among the medicinal plants, aromatic herbs are a rich source of biologically active compounds useful both in agriculture and medicine (Mathela, 1991; Cutler and Cutler, 1999). Two types of *Ocimum* are met within cultivation: (i) with green leaves known as Rama or

Lakshmi/Light tulsi or *Ocimum tenuifolium* and (ii) with purple leaves known as Krishna/Syama/Dark tulsi or *O. tenuiflorum* (Pandey, 1990). The chemical composition of Tulsi is highly complex, containing many nutrients and other biologically active compounds, the proportions of which may vary considerably between cultivars and even among plants within the same field. Further more the quantity of many of these constituents are significantly affected by differing growing, harvesting, processing and storage conditions that are not yet well understood.

In Ayurveda, this tulsi plant has been well documented for its therapeutic potentials and described as Dashemani Shwasaharni (antiasthmatic) and antikaphic drugs (Kaphaghna) (Sirkar, 1989). Some of biological properties is well recorded by various researchers, Anticancer properties (Kathiresan, Guanasekan, Rammurthy, & Govidswami, 1999), radioprotective, anticarcinogenic (Devi, 2001), antioxidant (Devi, 2001; Joshi, 2013a), chemopreventive, immunotherapeutic (Mukherjee, Das, & Ram, 2005), antimicrobial (Singh, Malhotra, & Majumdar, 2005; Joshi, 2013a), anti-inflammatory (Godhwani, Godhwani, & Vyas, 1987; Singh & Majumdar, 1997), analgesic, antipyretic (Godhwani *et al.*, 1987), antispermatic and antistress (Bhargava and Singh, 1981). The main aim of this study was to investigate and compare few aspects of phytochemical, antimicrobial and antioxidant studies between two lamiaceae species *O. tenuifolium* L. and *O. tenuiflorum* L. collected from the regions of Visakhapatnam, Andhra Pradesh, India.

Materials and Methods

Plant materials

Collected the plants *Ocimum tenuiflorum* and *Ocimum tenuifolium* from the regions of Visakhapatnam, Andhra Pradesh, India, were free from diseases. These plant parts were cleaned of residual soil and air-dried at room temperature.

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Solvents and chemicals used

All chemicals were purchased from Qualigens fine Chemicals, Mumbai and S.D. fine chemicals, Mumbai. microbial culture media and antibiotics used in this study were procured from Hi Media, Mumbai, India.

Microorganisms

Seven human pathogenic microorganisms were selected to screen the antimicrobial activity of the selected plant extracts, of these four were bacteria and three were fungal common dermatophytes in human listed in Table 1.

Table 1: List of Microorganisms

Micro Organism	Type	MTCC No.
<i>Escherichia coli</i>	Bacteria	443
<i>Proteus vulgaris</i>	Bacteria	1771
<i>Klebsiella pneumonia</i>	Bacteria	530
<i>Staphylococcus aureus</i>	Bacteria	3381
<i>Trichophyton mentagrophytes</i>	Fungi	7687
<i>Epidermophyton floccosum</i>	Fungi	613
<i>Candida albicans</i>	Fungi	227

Preparation of plant extracts

The shade dried plant materials were coarsely powdered by using pulverizer. Coarsely powdered material weighed and extracted with respective solvents hexane, chloroform and methanol using a soxhlet extractor for five to six hours at temperature not exceeding the boiling point of the solvents. For each gram of dry material 2ml of solvents was used. The extracted solvents were filtered through Whatman no-1 filter paper and subsequently concentrated under reduced pressure (in vacuum at 40°C) using a rotary evaporator. The residue obtained was designated as crude extracts, were labeled and stored in refrigerator for further study (Nostro et al., 2000).

Preliminary phytochemical investigation

The extracts of phytochemical analysis for identification of bioactive chemical constituents were carried out by using standard methods of Sofowora, Trease & Evans, Kokate, Harbone and Raman (Sofowora, 1993; Trease and Evans, 1989; Kokate, 2005; Harbone, 1984; Raman, 2006).

Test for Terpenoids: To 1-2 ml of all the extracts 1% HCl was added and allowed to stand for 5-6 hours. Later, these extracts were treated with 1ml of Trim-Hill reagent (a solution of 10 ml of acetic acid, 1 ml of 0.2% copper sulphate in water and 0.5 ml of concentrated hydrochloric acid) and heated in a boiling water bath for 5-10 minutes. Formation of bluish green color indicates the presence of terpenoids.

Test for Quinones: The extracts were treated separately with Alc. KOH solution. Appearance of colors ranging from red to blue indicates the presence of Quinones.

Test for Coumarins: 1-2ml of all the extracts were taken in separate tubes and covered with a piece of paper soaked in NaOH and heated. When these tubes yield a yellow fluorescence under UV light indicates the presence of coumarins.

Test for glycosides:

2-3 drops of molish reagent was added to the extracts and mixed well. To this, few drops of conc. H₂SO₄ was added carefully. Formation of reddish-purple colored ring at the junction of two layers indicates the presence of glycosides.

Identification alkaloids by precipitation method:

2mgs of KI and 1.25gms of iodide are dissolved in 100ml of distilled water. When the alkaloid extract is

treated with Wagner's reagent, brown or reddish-brown color precipitate obtained.

Detection of Flavonoids

Test solution when treated with few drops of FeCl₃ would result in the formation of blackish red color indicating the presence of flavonoids.

Detection of Steroids

2ml of acetic anhydride was added to 0.5g ethanolic extract of each sample with 2ml of H₂SO₄. The color changes from violet to blue or green indicates the presence of steroids.

Detection of Tannins

To the 1ml of plant extract, few drops of 1% FeCl₃ solution were added. The appearance of blue, black, green or blue green precipitate indicates the presence of tannins.

Detection of Phenols

To the 1ml of plant extracts, 3ml of distilled H₂O was added. To this few drop of neutral 5% FeCl₃ solution were added. A dark green color indicates the presence of phenols.

Detection of Saponins

About 2ml of distilled H₂O and 1ml of plant extract were mixed and shaken vigorously. A stable persistent froth indicates the presence of Saponins.

Detection of Cardiac Glycosides

Plant extract was dissolved in glacial acetic acid containing traces of FeCl₃. Then the tube was held at an angle of 45°, 1ml of conc. H₂SO₄ was added down the side purple ring at the interface indicates cardiac glycosides.

In vitro antimicrobial assays:

The development of simple *in vitro* pre-screens could offer initial idea of the biological activity of plant extracts and its compounds. Agar well diffusion assay was used to screen for the antimicrobial activity of extracts of different plant species. Which is the most widely used type for identifying the antimicrobial activity, which exploit diffusion of antimicrobial compounds through agar media to demonstrate the inhibition of bacteria and fungi.

Agar well diffusion method:

In agar well diffusion method peptone (0.5grams), meat extract (1.0 grams), sodium chloride (0.5 grams) and agar (1.5grams) were dissolved in small quantity of distilled water with the aid of heat on water bath and the volume was made up to 100 ml with purified water. The pH of the nutrient broth was adjusted to 7.2 using 5M sodium hydroxide, and then sterilized in an autoclave maintained at 121°C (15lbs.) for 20 minutes. After sterilization, the medium was inoculated with 3μl aliquots of culture containing approximately 10⁵ CFU/ml of each organism of 24 hours slant culture in aseptic condition and transferred into sterile petri dishes and allowed to set at room temperature for about 10 minutes and then kept in a refrigerator for 30 minutes. After setting a number 3 cup borer (6mm) diameter was properly sterilized by flaming and used to make four to five uniform cups/wells in each petri dish. A drop of molten nutrient agar was used to seal the base of each cup. The cups/wells were filled with 50μl of the different extracts of 100mg/ml, 250mg/ml, and 500mg/ml so final drug concentration will be 5mg/well, 15mg/well, and

25mg/well respectively and allow diffusing of plant extract into the medium for about 45 minutes. Standard drugs ciproflaxacin (10µg/ml), control (0.1% DMSO) were transferred to the cups of each agar plate by means of sterile pipettes under a laminar flow unit. The plates thus prepared were left for 2 hours in refrigerator for diffusion and then kept in an incubator at 37°C. After 24 hours, the agar plates were examined for inhibition zones, and the zones were measured in millimeters. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates.

In vitro Antioxidant activities

Ferric Reducing Antioxidant Power (FRAP) Assay

Total antioxidant potential of hexane, chloroform and methanol extracts were determined using FRAP assay. An amount of 200µl extracted samples were mixed with 3ml FRAP reagent in test tubes and vortexed. Blank samples were prepared for both methanol and deionized water extracted samples. Both samples and blank were incubated in water bath for 30 minutes at 37°C and the absorbance of the samples was determined against blank at 593nm. Series of stock solution at 200, 400, 800, 1200 and 1600µM were prepared ($r^2 = 0.9944$) using aqueous solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ as standard curve. The values obtained were expressed as µM of ferrous equivalent Fe (II) per gram of freeze-dried sample.

DPPH Radical Scavenging Assay

Plant extracts were tested for the scavenging effect on DPPH radical method, 2ml of extract solution of different solvents (Hexane, Chloroform and Methanol) were taken in different concentration to which 2 ml of 0.4 mM/L DPPH methanolic solution was added. Solution containing 2ml of methanol and 2ml of the DPPH solution was used as negative control and synthetic antioxidant ascorbic acid was used as positive control. Different concentrations were kept in the dark at room temperature for 30 min. The scavenging activity of the DPPH was determined by measuring the absorbance at 517 nm until the reaction reached the steady state, using a spectrophotometer. All the determination was performed five times. The DPPH radical scavenging activity was calculated using the following equation.

$$\% \text{ inhibition} = (1 - A_1/A_0) \times 100$$

A_1 and A_0 are the absorbance of the tested sample and control respectively.

Results and Discussions

Preliminary phytochemical investigation of the Hexane, Chloroform and Methanol extracts of *O. tenuiflorum* L. and *O. tenuifolium* L. (were compared). The results of the phytochemical screening of hexane, chloroform and methanolic extract of both *Ocimum* species, revealed the presence of terpenoids, alkaloids, steroids, saponins and tannins compounds (Table 2). Terpenoids and Alkaloids were present in the extract of Chloroform, Cardiac glycosides were evident in Chloroform, Hexane. It also shows that Tannins were present in Aqueous, Chloroform and Methanol soluble extracts only. Cardiac Glycosides were only evident in Chloroform and Hexane extracts whereas Resins and Steroids were absent in all the compared extracts of the plants. This presence of compounds has significant application against several pathogens and therefore could suggest their use in the treatment of various diseases.

The presence of these phytochemical components may be responsible for the observed antibacterial activity of the plant leaf extract. Flavonoid has also been reported to have greater potential benefit to human Health. The beneficial medicinal effects of plant materials typically result from the secondary metabolites present in the plant although, it is usually not attributed to a single compound but a synergetic result. The medicinal properties of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh *et al.*, 2005). It is necessary to investigate those plants scientifically which have been used in traditional medicine to improve the quality of healthcare. Plant glycosides, which are not normally toxic when ingested orally, are known to inhibit chloride transport in the stomach (Machen and Forte, 1979). Awareness of local community should be enhanced incorporating the traditional knowledge with scientific findings.

Table 2. Qualitative phytochemical analysis

Test	Hexane		Chloroform		Methanol	
	<i>O.tenuiflorum</i>	<i>O.tenuifolium</i>	<i>O.tenuiflorum</i>	<i>O.tenuifolium</i>	<i>O.tenuiflorum</i>	<i>O.Tenuifolium</i>
Alkaloids	-	+	-	+	+	+
Coumarins	+	-	+	+	-	+
Flavonoids	+	+	+	+	+	+
Glycosides	-	-	-	-	+	-
Phenols	-	-	+	-	+	+
Quinones	+	-	-	-	+	-
Reducing sugar	-	+	+	+	+	+
Steroids	+	-	-	+	+	+
Saponins	-	-	-	-	+	+
Tannins	-	-	+	+	+	+
Terpenoids	-	+	+	+	+	+

The preliminary phytochemical parameters were studied not only in search of bioactive agents but also for starting products which uses in the synthesis of useful drugs (Vimal, *et al.*, 2012). The *Ocimum* species were broadly used for the treatment of different diseases in third world countries; in the latest research both *Ocimum* sps: found that it may have natural bioactive compounds which provide protection to animal against different diseases [Vivek, *et al.*, 2006].

Table 3. Ferric Reducing Antioxidant Power (FRAP) assays on selected medicinal plants

S.No.	Medicinal Plant	Hexane	Chloroform	Methanol
1	<i>O. tenuiflorum</i>	35.5	30.6	78.5
2	<i>O. tenuifolium</i>	25.2	47.3	69.6

Result expressed in µg/ml

Table 4. *In vitro* concentration represented by percentage inhibition of DPPH radical with plant extract/ Ascorbic acid in micrograms (µg/mL)

S.No.	Plant Name	Hexane (%)	Chloroform (%)	Methanol (%)
1	<i>O. tenuiflorum</i>	43.64 ± 1.94	57.50 ± 1.21	69.10 ± 0.21
2	<i>O. tenuifolium</i>	23.33 ± 0.56	49.76 ± 0.19	77.25 ± 0.21

Table 5. Anti-microbial activity *O. tenuiflorum*

Micro-Organism	Methanol				Control
	100	250	500	Standard Ciprofloxacin	
<i>E. coli</i>	9	12	16	40	NA
<i>K. pneumonia</i>	8	9	12	34	NA
<i>S. aureus</i>	10	11	13	39	NA
<i>P. vulgaris</i>	10	13	15	33	NA
<i>T. mentagrophytes</i>	NA	NA	9	40	NA
<i>E. floccosum</i>	NA	8	10	33	NA
<i>C. albicans</i>	NA	9	12	37	NA

NA-No Activity; Zone of Inhibition in mm

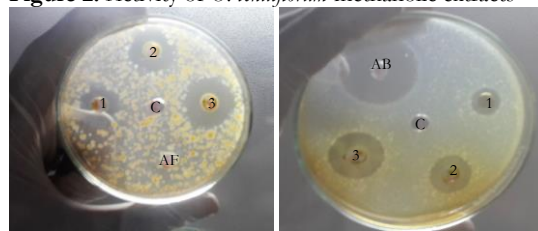
Table 6. Anti-microbial activity *O. tenuiflorum*

Micro-Organism	Methanol				Control
	100	250	500	Standard Ciprofloxacin	
<i>E. coli</i>	8	11	13	40	NA
<i>K. pneumonia</i>	10	11	15	34	NA
<i>S. aureus</i>	NA	8	10	39	NA
<i>P. vulgaris</i>	9	9	13	33	NA
<i>T. mentagrophytes</i>	8	9	11	40	NA
<i>E. floccosum</i>	9	11	13	33	NA
<i>C. albicans</i>	11	14	16	37	NA

NA-No Activity; Zone of Inhibition in mm

Figure 1. Activity of *O. tenuiflorum* methanolic extracts

S. aureus *E. coli*
1-100; 2-250; 3-500mg/ml; C-Control; AB-Antibiotic;
AF-Antifungal drug

Figure 2. Activity of *O. tenuiflorum* methanolic extracts

Candida albicans *Klebsiella pneumonia*
1-100; 2-250; 3-500mg/ml; C-Control; AB-Antibiotic;
AF-Antifungal drug

Discussions

Antimicrobial activity from plant sources can be assumed to be useful. Medicinally, this is important for the treatment of inflamed tissues (Mota MLR, et al., 1985). The chloroform extracts and methanol extracts of *O. tenuiflorum* and *O. tenuifolium* have inhibited the growth of Gram negative and Gram-positive bacteria. The results reveal the presence of medicinally active constituents in the leaves of these plants can be used in medicine as anesthetic agents (Victor Njoku O. and Chidi Obi, 2009). The results obtained in this study thus suggest that the identified phytochemical compounds may be the bioactive constituents responsible for the efficacy of the plants studied. The presence of some of these compounds has also been confirmed to have antimicrobial activity. Hence it could be inferred that the plant extracts could be a source for the industrial manufacture of drugs useful in the chemotherapy of some microbial especially fungal infection.

The results indicated that from *O. tenuiflorum* and *O. tenuifolium* extract showed effective antibacterial activity both in Gram negative and Gram-positive bacteria by good inhibition against the studied bacterial strains than three fungal strains. Methanol extracts exhibited greater microbicidal as well as antioxidant studies values when compared with chloroform and hexane extracts. So only chloroform/methanol activities were reported. This observed antimicrobial activity could be explained by the fact that plant extract may attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell. The interaction of plant extract

phenolic terpene constituents with microbial cytoplasmic components and nucleic acids can inhibit the respiratory chain enzymes, and interferes with the membrane permeability, limiting the development of bacteria and yeasts. It is also possible that extract not only interact with the surface of membrane, can also penetrate inside the bacteria. The susceptibility of Gram positive and Gram-negative bacteria to extract was found to vary from one study to another. Whereas Antony et al., reported that extract had a considerably minimal microbicidal activity on Gram positive bacteria compared to Gram negative bacteria which they attributed to the high lipo polysaccharide and thick peptidoglycan layer of the microorganisms. Present investigation revealed that both *Ocimum* sp. exerted significant antimicrobial activity against both bacteria and fungal strains.

Conclusion

In this study the *O. tenuiflorum* and *O. tenuifolium* were shown significant antimicrobial activity. Because of the presence of eugenol like phenolic terpenes. those are more potential to anti-microbial properties. which can be easily accessible source of natural anti-microbial and as well as a possible food preservative, and is one of the most important plant in pharmaceutical industries

It is therefore confirmed as a useful antimicrobial agent. And also have free radical scavenging activity so called anti-oxidants. It is strongly believed that detailed information has presented in this review on the phytochemical and various biological activities of the extracts might have provide detailed evidence for the use of this plant in different medicines. The flavonoids in leaves 0.50mg/g and in stem 0.60mg/g which was also greater than stem. The presence of terpenoids and flavonoids confirms that the plant has higher antioxidant value, as well as justify its antimicrobial, anti inflammatory, antimutagenic, antiviral and anti allergic actions. Both *Ocimum* Sps. have almost same nutritional, minerals and phytochemical values. The present relative study on these two *Ocimum* sps. revealed that *O. tenuiflorum* had higher biological potential than the other sps. studied. Therefore, both the plants can be used in traditional medicine to control different types of ailments.

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