ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF OCIMUM SANCTUM(P) LEAF ESSENTIAL OILS.

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Received: Sep. 2019 Accepted: Oct. 2019 Published: Feb. 2020

Abstract: *Ocimum sanctum* also known as *Ocimum tenuiflorum* is commonly known as *Tulsi* or *holy basil* or, the Queen of herbs.There are two kinds of *Ocimum sanctum* available; they are Red or Purple variety called Krishna Tulsi and White or Green variety called Sri Tulsi. Eugenol is the major constituent present in this plant(61.538%). Extracts from the leaves of *Ocimum sanctum* purple were investigated for phytochemical constituent antioxidant and antomicrobial activity. Tests for phenols, flavonoids and antioxidants of *Ocimum sanctum* (P)were conducted and the value of phenols found to be 127.3±1.8mg/gm. The value was compared with that of tannic acid. The flavonoids content of *O.sanctum* was 0.733±0.01mg/gm . The value was compared with quercetin. The antioxidant content was evaluated by using ascorbic acid as standard and the value was 0.921±0.03 mg/gm. The percentage inhibition of DPPH (scavenging activity) was 46.91% . Antibacterial activity by disc diffusion method reveals that essential oils of *Ocimum sanctum* (P)plants was active against the gram positive bacteria (*Staphylococcus epidermidis, Staphylococcus aureus*, *Bacillus paludis*, *Bacillus subtilis*) the gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa,* and *Shigella flaxinely*, *Enterobacter aerogenes*) and fungus (*Candida albicans*, *Aspergillus fumigatus*, *Aspergillus niger*) This study revealed that extract of leaves *Ocimum sanctum*(P*)* plant comprises effective potential source of natural antioxidants and also antimicrobial activity due to the presence of Eugenol.

Keywords: Ocimum Sanctum/Tenuliflorum, Purple, % Eugenol, Total Phenols, Flavonoids, Antioxidants, DPPH Assay, Antimicrobial Activity.

1. Introduction: *Ocimum sanctum* also known as *Ocimum tenuiflorum* belongs to Lamiaceae family is commonly known as *Tulsi* or *holy basil.* The plant, which is native to tropical Asia, is now found in most tropical parts of the world. The plant grows wild in India but is also widely cultivated in home and temple gardens and is used for household remediation [1]. *Holy basil* has a strong anise like, slightly musky and lemony taste with a camphoraceous aroma. The dominant aroma component in *holy basil* is Eugenol. There are two kinds of *holy basil*: the more exuberantly flavoured red *holy basil* (red or purple variety) has dark green leaves with reddish purple stems and a purplish cast on the younger leaves known as *Krishna Tulsi*, while the milder white (white or green variety) has medium green leaves with very light green, almost white stems known as *Sri Tulsi*[2], their chemical constituents are similar[3], and also have common medicinal properties[4].The stem and leaves of holy basil contain a variety of constituents that may have biological activity, including saponins, flavonoids, triterpenoids, and tannins[5]. The leaf volatile oil[6] contains eugenol, euginal (also called eugenic acid), urosolic acid[7], carvacrol, linalool, limatrol, caryophyllene, methyl carvicol, uteolin, vitexin, isovitexin, orientin, isoorientin, aesculin, chlorogenic acid, aesculetin, caffeic acid[8], betacarotene[9], Rosmarinic acid, apigenin, cirsimaritin, isothymusin and isothymonin. Two watersoluble flavonoids:[10] Orientin and Vicenin, ascorbic acid and carotene are also reported[11]. Different parts of *Tulsi* plant such as leaves, flowers, stem, root, seeds etc. are known to possess therapeutic potentials and have been used by traditional medical practitioners, as expectorant, analgesic, anticancer, antiasthmatic, antiemetic,

diaphoretic, antidiabetic, antifertility, hepatoprotective, hypotensive, antistress, analgesic, anti-hyperlipidemic, antioxidant potentials in experimental animals[12-17]. The objective of this research was to examine the % composition of eugenol, total phenols, flavonoids , antioxidants, DPPH assay and antimicrobial activity of *O.sanctum*(P).

2. Materials and Methods

2.1 Collection and Identification of Plant Material: The selected plants were grown in the same geographical conditions in the spacious domestic home garden at Gavaravaram village, Eluru mandal in West Godavari district of Andhra Pradesh is situated between 16.7° North 81.1° East, elevation 22mts 72 feet. Collections (leaves) were made. Voucher of specimen was deposited at the Botany department. In the present study the specimen number was N2. The specimen was subjected for identification at plant systematic laboratory, Kakatiya University, Warangal, Andhra Pradesh, India.
1 Kg of leaves of the sample was collected in the month of November 2010. The sample was dried under shade, grinded to fine powder in an electric blender (80 mesh) and stored in air tight container at room temperature in the dark until used.

2.2 Preparation of Plant Extract: One gram of dried leaf powder was grinded with 20ml of 50% methanol and filtered. The filtrate was made up to the volume 50ml with 50% methanol. This extract was used to analyze the total phenol content, the flavonoids content and the antioxidant capacity.

2.2.1 Soxhlet Extraction: Extraction of total essential oil content of plant materials was carried out by soxhlet extraction[18] method. 5gm of dry powder was subjected to soxhulet extraction with 250ml methanol, extraction was carried out for 3hrs, 10 cycles and temperature was maintained at 65°C.This extract was used to analyze DPPH assay and the antimicrobialactivity.

2.2.2 Steam Distillation: Extraction of volatile oils from the plant materials was carried out by steam distillation using Clevenger type apparatus[19]. 100g powdered sample was water distilled by using a Clevenger oil arm fitted with condensers through which cooled water was circulated to prevent low volatiles from escaping. The temperature was maintained at 60°C. The volatile oil was collected and dried over anhydrous Sodium Sulphate and stored at -4 °C. 1mg of volatile extract was dissolved in 1ml of methanol, from that solution 10µl was taken and made up to 100 µl with methanol. This solution was used for GC analysis. (Same procedure followed for the preparation of standard eugenol).

2.3 Gas Chromatography Analysis: The essential oils were analyzed using a Shimadzu gas chromatograph model 17 A Japan(2014), equipped with flame ionization detector (FID) and DB-Wax capillary column (30mx0.32mm , film thickness 0. 5 µm). Injector and detector temperatures were set at 240 and 250°C, respectively. Column oven temperature was programmed from 40°C to 220°C at the rate of 8°C min-1; initial and final temperatures were held for 3 and 10 minutes, respectively. Helium was used as a carrier gas with a flow of 1.5 mL min-1. A sample of 0.1 µL was injected using slit mode (split ratio, 1:20). Quantification was completed by built-in data-handling software supplied by the manufacturer (spin chrome CFR) of the gas chromatograph. The results (composition) were reported as a relative percentage of the total peak area.

2.4 Estimation of Total Phenolics: Total phenolic content was determined using Folin-Ciocalteau reagent as previously described[20]. The plant extract solution (250 µl) was mixed with 5ml of Folin-Ciocalteu reagentand 4 ml of (20%) sodium carbonate, and they were vortexed for 50sec and they were let to stand for 30mins in water bath at 40°C. The optical density was measured by using systronics (C1117) colorimeter using at 680nm. The total phenol content of the extracts was obtained by using the standard curve. The total phenol content was expressed as tannic acid (0.1mg/ml) equivalent in % w/w of the extracts.

Total Phenolic content = $\frac{Optical\ density\ of\ sample \times Concentration\ of\ tannic\ acid}{Optical\ density\ of\ standard}$

2.5 Total Flavonoids Content: The total flavonoid content was determined using the Dowd method[21]. The plant extract solution (250 µl) was mixed with 0.1ml of 2% aluminium chloride and 1ml

of 0.1M potassium acetate, mixed well and allowed to stand for 30min. at room temperature. The colour developed in each test tube was measured by using systronics (C1117) colorimeter at 420nm. Total flavonoid contents were calculated as quercetin(0.1mg/ml) equivalent from a calibration curve.

$$\text{Total Flavonoid content} = \frac{Optical\ density\ of\ sample \times Concentration\ of\ Quercetin}{Optical\ density\ of\ standard}$$

2.6 Reducing Power Assay: The reducing power of the extracts was measured by using ascorbic acid[22]. The plant extract solution (250 µl) was mixed 2.5 ml of phosphate buffer (PH 6.6) and 2.5ml of(1%) potassium ferricyanide and were incubated in water bath at 50°C for 20min. Then the test tube was centrifuged for 10min. at 10,000 rpm. 2.5ml of supernatant was taken in a test tube 2.5ml of distilled water and 0.5ml freshly prepared (0.1%)ferric chloride were added and observed the colour change. The optical density was measured by using UV–VIS spectrophotometer 2.2. (Double-beam) (SL191 series) at 680nm. Total antioxidants contents were calculated as ascorbic acid (0.1mg/ml) equivalent from a calibration curve.

$$\text{Total antioxidants content} = \frac{Optical\ density\ of\ sample \times Concentration\ of\ ascorbic\ acid}{Optical\ density\ of\ standard}$$

2.7 DPPH radical scavenging assay (Antioxidant assay)

The following assay procedure was modified from those described by Blois[23]. and Govindaragan[24]. Sample stock solutions (1.0 mg/ml) were diluted to final concentrations of 250, 125, 50, 25, 10 and 5 µg/ml in methanol. A portion of sample solution (500 µl) was mixed with an equal volume of 6x10-5 M DPPH (1,1- diphenyl-2-picrylhydrazyl; in methanol) and allowed to stand at room temperature for 30 min. In each experiment methanol (1ml) plus plant extract solution was used as blank while the DPPH solution alone in methanol was used as control. The absorbance (A) of sample solution was measured in spectrophotometer at 520 nm, compared with that of control solution (maximum absorbance). The scavenging activity of samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage inhibition.

$$\%\ \text{Inhibition} = \frac{[(A\ Control - A\ Sample)]}{A\ Control} \times 100$$

2.8 Agar Disc Diffusion Method: The bacterial strains used were the gram positive bacteria, *Staphylococcus epidermidis Staphylococcus aureus,* B*acillus paludis, Bacillus subtilis,* the gram negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Shigella Flaxinely, Enterobacter Aerogenes*. The fungus used was *Candida albicans, Aspergillus Fumigatus , Aspergillus Niger.* The organisms were obtained from Institute of Microbial Technology, Chandigarh, India.

The antimicrobial assay was performed by the agar disc diffusion method [25-28]. In brief, the microbial suspension containing 108CFU/ml of bacteria was swabbed and spread on Muller- Hinton agar. The essential oil (20 µg) was applied on paper disc (6 mm in diameter) and placed on the inoculated agar. Neomycin (20 µg/disc), amoxicillin (20 µg/disc), were used as positive control standards to determine the sensitivity of gram negative bacteria and gram positive bacteria respectively. The inoculated plates were incubated at 37°C for 24h. The antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters (including disc diameter of 6 mm) against the test organisms and comparing to the controls.

The microbial suspension containing 108 CFU/ml of fungus was swabbed and spread on Sabouraud dextrose agar. The essential oil (20 µg) was applied on paper disc (6 mm in diameter) and placed on the inoculated agar. Clotrimazole (20 µg/disc) was used as positive control standards to determine the sensitivity of fungus. The inoculated plates were incubated at 37°C for 24h. The antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters (including disc diameter of 6 mm) against the test organisms and comparing to the controls.

3.Results and Discussion: In the present study several phytochemical constituents present in *Ocimum sanctum* purple such as total phenols, flavonoids and antioxidants were evaluated. The free radical

scavenging activity and antimicrobial activity of *Ocimum sanctum* was also evaluated.Table 1 shows the Percentage composition of eugenol, Total phenols, flovonoids, antioxidants and DPPH scavenging assay in *O. sanctum* Purple .

In *O. sanctum* purple have been found to yield 61.538% of eugenol. The percent of eugenol in essential oil of *O. sanctum L.* Varies in India varies from 40% (in Jammu) to 71% (in Assam) [29]. Asha and coworkers[30] reported that the oil of *ocimum sanctum* posses Eugenol(53.10%) as the main compound. The total phenol content of purple *holy basil* was 127.3± 1.8 mg tannic acid equivalent per gram of dry weight . Due to the higher phenol content in the leaf, the plant possesses high antioxidant activity and other pharmacological activities[31].

Table 1: Percentage composition of eugenol, Total phenols, flovonoids, antioxidants and DPPH scavenging assay in *O. sanctum* Purple

Name of the plant	Percentage composition of eugenol	Totalphenols mg TA equivalent / gm dw	Total flavonoids mg of quercetin/gmdw	Antioxidants mg of ascorbic acid equivalent /gm dw	DPPH scavenging % inhibition
Ocimum sanctum purple	61.538%	127.3±31.8	0.733±0.01	0.921±0.03	46.91%

Values are expressed as mean ± SD (n = 3). dw: dry weight of the powdered sample
The total flavonoids content of purple *holy basil* was 0.733±0.01 mg of quercetin equivalent per gramof dry weight . From these studies it was found that not only phenols flavonoids also contribute the antioxidant activity.

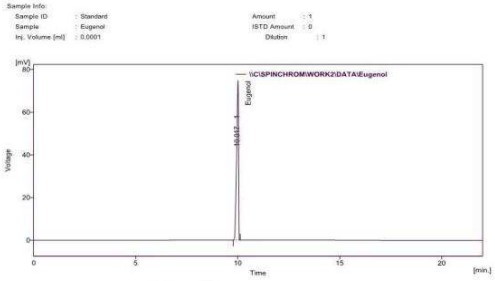
The antioxidants content of *Ocimum sanctum* purple was 0.921±0.03 mg of ascorbic acid equivalent /gm dw. *Ocimum sanctum* purple posses higher antioxidant activity due to the presence of high eugenol content[32]. In the present study it was found that there is a positive relationship between total phenols and antioxidant activity. The phenolic compounds may contribute directly to the antioxidant action[33]. The phenols contain hydroxyls that are responsible for antioxidant activity and the radical scavenging effect mainly due to redox properties [34-36].

The high inhibition of DPPH activity of *O. sanctum* purple in this recent study (46.91%) was lower than the value (71%) reported by Lukmanul Hakkim [37].

In *Ocimum sanctum* purple the zone of inhibition ranges from10.5-17mm and the high zone of inhibition was in *Bacillus paludis* and *Bacillus subtilis* (17mm). The essential oils of *Tulsi* have been effective against both Gram- positive and Gram-negative bacteria and the properties were comparable with the effectiveness of clove oil[38,39] .The essential oils from fresh leaves had shown more antibacterial properties compared to the essential oils from dried leaves of *Tulsi* and in case of fungus the property is just the reverse[40].

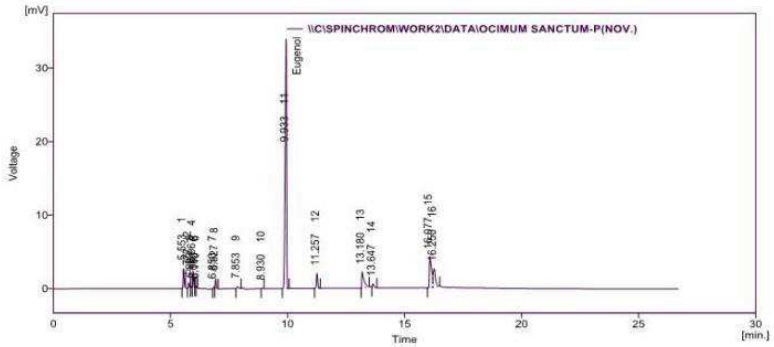
The ethanol and methanol extracts of *O.sanctum* had the ability to inhibit the growth of all test bacteria including *E.coli* and *P.aeruginosa*.[41] . Antimicrobial activity of *Ocimum sanctum* was found to be higher as compared to commonly available other species of *Ocimum* (*i.e. O. canum, O. gratissimum, O. basilicum*) in India[42] . The highest antimicrobial activity of essential oil of *O. sanctum* may be attributed to high percentage of eugenol[43]. The antifungal properties have been reported from the essential oil of *O. sanctum*.[44-46].

Standard Eugenol- Chromatogram



Result Table (Uncal - \\C\SPINCHROM\WORK2\DATA\Eugenol)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	10.017	464.988	74.656	100.000
	Total	464.988	74.656	100.000

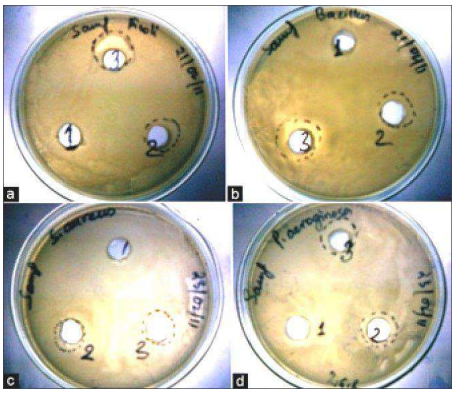


Result Table (Uncal - \\C\SPINCHROM\WORK2\DATA\OCIMUM SANCTUM-P(NOV.)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	5.553	8.377	2.704	3.489
2	5.777	2.342	0.774	0.975
3	5.893	0.418	0.212	0.174
4	5.967	5.769	2.193	2.402
5	6.060	0.154	0.093	0.064
6	6.110	0.250	0.111	0.104
7	6.850	0.727	0.274	0.303
8	6.927	4.012	1.391	1.671
9	7.853	1.360	0.286	0.566
10	8.930	0.298	0.094	0.124
11	9.933	147.773	33.853	61.538
12	11.257	7.366	2.043	3.067
13	13.180	14.971	2.203	6.234
14	13.647	2.109	0.485	0.878
15	16.077	26.262	4.194	10.937
16	16.250	17.946	2.494	7.473
	Total	240.135	53.404	100.000

Name of the organism	Ocimum Sanctum(p)
Staphylococcus Epidermidis(G+veB)	13
StaphylococcusAureus(G+veB)	16
Bacilluspaludis(G+veB)	17
Bacillussubtilis(G+veB)	17
Escherichiacoli(G-veB)	12
Pseudomonusaeruginosa(GveB)	12
Shigellaflaxinely(G-veB)	10.5
Enterobacteraerogenes(GveB)	11
Candidaalbicans(F)	13
AspergillusFumigates(F)	11
Aspergillusniger(F)	16

Table-2:Antimicrobial activity



Antimicrobial activity

Values are expressed as mean ± SD (n = 3).
G+veB= gram positive bacteria, G-veB= gram negative bacteria, F= fungus

4. Conclusions: Eugenol, (l-hydroxy-2-methoxy-4- allylbenzene), is a phenolic compound, major volatile constituent of essential oils extracted from leaves of the selected plant. The plant possessed significant antioxidant and antimicrobial activities, and it was mainly due to the presence of eugenol. This plant is easily available in our surroundings and the process of cultivation is also very easy. So we can make use of these plants to extract the costly component like eugenol. These plants show different medicinal properties. So, still more clinical trials should be conducted to support their medicinal therapeutic uses.

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