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RESEARCH ARTICLE

AN IN VITRO EVALUATION OF ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF ETHYL ACETATE EXTRACT OF *ORTHOSIPHON STAMINEUS*

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ABSTRACT

Objectives: The present study was visualised to identify the effect of antioxidant and antibacterial activity for ethyl acetate extract of *Orthosiphon stamineus.* **Methods:** The present study recorded to evaluate the *in vitro* antioxidant and antibacterial activity of ethyl acetate extract of *Orthosiphon stamineus.* To examine the extract tested against Gram positive and Gram negative MTCC bacterial species using the agar well diffusion and MIC method. *In vitro* antioxidant activity was evaluated using ABTS radical scanning, lipid peroxidation and metal chelating assay method. **Results:** The ethyl acetate extract from the leaves of *O. stamineus* was active against all tested bacterial strains specifically *Escherichia coli* (17.8 mm) and *Staphylococcus aureus* 16.3 mm inhibition were recorded. Total polyphenols and flavonoids contents, as well as antioxidant activities like ABTS radicals (66.23%), inhibition of lipid peroxidation (81.23%) and nitric oxide scavenging (83.21%) were performed by ethyl acetate extract from the leaves of *O. stamineus*. **Conclusion:** The ethyl acetate extract from the leaves of *Orthosiphon stamineus* are suitable candidates for the development of novel antibacterial and antioxidant compounds.

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INTRODUCTION

Plant extracts familiarized into food positively touch its organoleptic properties, giving the foodstuffs a specific taste and smell, at the similar stretch are a source of natural substances comprising bioactive compounds, among others polyphenol compounds with antioxidant properties. The antioxidant properties of extracts can affect the extension of shelf life and in many cases improve the microbiological quality of food. Research is increasingly pointing to the possibility of using antimicrobial and antioxidant properties of medicinal plants extracts and their starter into food products as natural preservatives. Plant tissues contained rich secondary metabolism are naturally in nutritive or purifyingly bioactive materials. Many researchers have reported that phenolic compounds and polyphenol extracts derived from medicnal plants can delay age-related decline and extend lifespan across a variety of species (Wereńska, 2013). The diet rich in polyphenols has been related with dropping the risk for cardiovascular diseases, cancer, and other age factor diseases. The microbial organisms including Gram positive and Gram negative like different species of *Klepsella*, *Staphylococcus, E.coli* and *Pseudomonas* are the main source to cause severe infections in humans. For the reason that these organisms have the capacity to persist in harsh condition due to their manifold environmental condition. The synthetic antibiotics have the following limitation: Firstly, these are costly and are out of range from the patient belonging to developing countries. Secondly, with the passage of time microorganism develop resistance against antibiotics. Therefore, after some time these antibiotics are not effective against the microbes (Kaur and Kapoor, 2001). Furthermore, the antibiotics may be associated with adverse effects on the host, including hypersensitivity, immune suppression, and also allergic reactions. On the other hand, natural products have got incredible success in serving as a guidepost for new antibacterial drug discovery (Henie *et al*., 2009). Orthosiphon stamineus Benth. (Lamiaceae) is a valued medicinal plant in traditional folk medicine. Many pharmacopoeias such as Indian, Indonesian and Swiss have listed this plant for the treatment related to renal cleansing and function, and related disorders that include nephritis, cystitis, and urethritis. In Europe, people use the leaves of O. stamineus extract as a tonic for kidney and bladder stones, liver and gallbladder problems, and urinary tract infections. This can be used to reduce cholesterol and blood pressure. Earlier report showed that this plant contains high amount of flavones, polyphenols, bioactive proteins, glycosides, a volatile oil, and vast quantities of potassium (Eisai, 1995).

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MATERIALS AND METHODS

PLANT COLLECTION AND PREPARATION OF EXTRACTS: *Orthosiphon stamineus* leaves was obtained from Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai, Tamilnadu, India. A plant taxonomist authenticated the plant and samples were kept in the Medicinal Botany herbarium with voucher specimen numbers MB/GSMC-396/2021.

PHYTOCHEMICAL ANALYSIS: The aqueous extract of *Orthosiphon stamineus* were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Harborne 1973; Trease and Evans 1983).

PREPARATION OF PLANT EXTRACTS: The leaves of *Orthosiphon stamineus* were dried in the laboratory excluding direct sun light at room temperature for 6-7 days and ground into a fine powder by mortar and pestle. Approximately, 100 g of plant powder was extracted with 500 ml of ethyl acetate for 24 h in a dark room temperature. Subsequently, the solvent was evaporated in a rotary vacuum evaporator at 40°C and re-suspended in dimethyl sulfoxide (DMSO). The homogenate was filtered through Whatman No. 1 filter paper, centrifuged at 5000 *g* for 15 min and sterilized by filtration through 0.22-µm sterile filters (Millipore, Bedford, MA, USA). The filtrates were stored at -4°C and used for *in vitro* screening of antimicrobial activity.

THIN LAYER CHROMATOGRAPHY: The ethyl acetate extract from the leaves of *Orthosiphon stamineus* were loaded on to pre coated TLC (60 F_2 54) and it was developed using solvent system in the ratio of Petroleum ether, Chloroform and methanol (1:0.5:0.1, V/V/V) was used for the development of the exudates on silica gel plates silica gel 60 F_{254} (10x20 cm, 0.2mmlayer). Visible and the non-visible spot given and it is fluorescent with UV light at 360nm and 240nm.

BACTERIAL STRAINS: Bacteria used for the determination of antibacterial activities were *Klebsiella pneumoniae* MTCC 1756 *Escherichia coli* MTCC 2130 *Staphylococcus aureus* MTCC 1954, and *Pseudomonas aeruginosa* MTCC 1456.

ANTIBACTERIAL ACTIVITY: The antibacterial activities of the polyphenol rich fraction were assayed using the disc diffusion method (Drago *et al.,* 1999). Bacteria were grown overnight on Mueller Hinton agar plates, five colonies were suspended in 5 ml of sterile saline (0.9%) and the bacterial population in the suspension was adjusted to ~$3x10^8$ CFU/ml. A sterile cotton swab was dipped into the suspension and the swab rotated several times with firm pressure on the inside wall of the tube to remove the excess fluid. The swab was used to inoculate the dried surface of MH agar plate by streaking four times over the surface of the agar, rotating the plate approximately by 90º to ensure an even distribution of the inoculums. The medium was allowed to dry for about 3 min before adding a sterile disc of 6 mm diameter. Each disc was placed firmly on to the agar to provide uniform contact with the bacteria. Bioactive compound (50 µg) was weighed and dissolved in 1 ml of 7% ethyl acetate. The different concentration of ethyl acetate extract from the leaves of *Orthosiphon stamineus* was introduced on to each disc and the control disc received only 7% ethanol. The plates were incubated at 37°C for 24 h and the inhibition zone was measured and calculated. The experiments were carried out in duplicate three times. The results (mean value, *n*=3) were recorded by measuring the zones of growth inhibition surrounding the discs.

MINIMUM INHIBITORY CONCENTRATIONS (MICS): The minimum inhibitory concentrations of the isolated compounds were determined by dilution method (Brantner and Grein, 1994). The strains were grown in Mueller Hinton broth to exponential phase with an *A*560 of 0.8, representing 3.2×10^8 CFU/ml. Different dilutions of the ethyl acetate extract from the leaves of *Orthosiphon stamineus* were prepared to give solutions of 25, 50, 75, and 100 µg/ml. 0.5 ml of each concentration was added into separate test tubes containing 4ml of MH broth inoculated with 0.5 ml bacterial suspension at a final concentration of 10^6 CFU/ml. Each MIC was determined from five independent experiments performed in duplicate. The tubes containing 4.5 ml of bacterial inoculates and 0.5 ml of 7% ethyl acetate used as bacterial control, 4.5 ml of uninoculated MH broth and 0.5 ml PBS served as a blank. The tubes were incubated at 37 ◦C for 18 h; inhibition of bacterial growth was determined by measuring the absorbance at 560 nm.

ABTS RADICAL SCAVENGING ACTIVITY: The ABTS scavenging activity was determined by the method of Re *et al*. (1999). The ABTS solution was produced by mixing 7.4 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) with 2.6 mM potassium persulfate and then diluted with phosphate buffer saline (pH 7.4) until the absorbance of the ABTS solution was 0.70 ± 0.03 at 732 nm. After reacting 950 µL of diluted ABTS solution with 50 µL of ethyl acetate extract from the leaves of *Orthosiphon stamineus* for 10 min in the dark, the absorbance was measured at 732 nm using a spectrophotometer (Deep vision). The ABTS radical scavenging activity was calculated as the ratio of the decrease in absorbance between the sample and no sample.

INHIBITION OF LIPID PEROXIDATION ACTIVITY: Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of the ethyl acetate extract from the leaves of *Orthosiphon stamineus* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

NITRIC OXIDE RADICAL SCAVENGING ACTIVITY: The ability of date seeds extract to scavenge nitric oxide radicals was examined using a Griess reaction based on Boora *et al*. (2014). Griess reagent has been prepared by combining the same amount of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulphanilamide, both prepared in 2.5% phosphoric acid. For the experiment, 500 µL of 10 mM sodium nitroprusside in phosphate buffered saline (pH 7.4), mixed with 1 mL of ethyl acetate extract from the leaves of *Orthosiphon stamineus* at different concentrations (25-100 µg/mL) were incubated for 150 min at 25 °C. After the incubation period, 1.5 mL of freshly prepared Griess reagent was added to the resulting mixture. Then, the absorbance of the mixture was measured at 546 nm. The control and standard were prepared in a similar manner as was done for the test samples using buffer and Trolox instead of extract. The nitric oxide scavenging capacity of each extract was calculated as follows:

$$\% \text{ NOsa} = (\text{Abs.control} - \text{Abs.sample}) \times 100 / (\text{Abs.control})$$

Where Abs control and Abs sample are the absorbance of buffer and the absorbance of sample extract/standard, respectively. Then EC_{50} value that represents the concentration of drug or extracts required to scavenge 50% of nitric oxide radicals was determined.

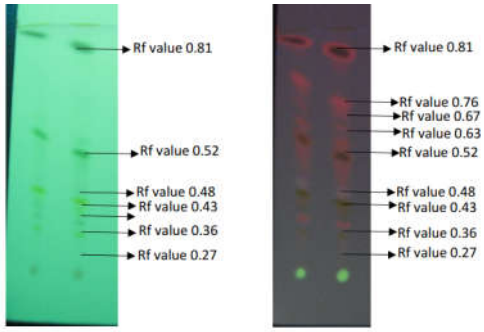
RESULT AND DISCUSION

PHYTOCHEMICAL SCREENING: The phytochemical screening of aqueous extract of *Orthosiphon stamineus* were studied presently showed the presence of alkaloids, flavonoids, polyphenol, terpenoids, and absence of glycosides (Table -1). Numerous researcher reported that *O. stamineus* leaves wing to rich phenol content and also more than 20 phenolic compounds isolated from this plant including lipophilic flavones, flavonol glycosides, and caffeic acid derivatives such as rosmarinic acid (Olah *et al*., 2003).

Table 1. Phytochemical screening of aqueous extract of *Orthosiphon stamineus*

Sl. No.	Phytochemical Constituents	Observation	Aqueous extract of *Orthosiphon stamineus*
1	Alkaloids - Dragendorff's Test -Mayers test	Orange / red precipitate Yellow or white precipitate	+ +
2.	Flavonoids -Alkalai Reagent -Lead acetate test	 Intense yellow colour Precipitate formed	 + +
3.	Glycosides Keller-Killiani test	Reddish brown colour ring formed	-
4.	Tannin -$FeCl_3$ test	Blue black coloration	+
5.	Saponins -Frothing test	Foam	+
6.	Terpenoids -Salkowski test	Dark reddish brown color in interface	+
7.	Polyphenols -Ferrozine test	Raddish blue	+
8.	Anthocyanin test Ammonia	Ammonia layer yellow in color	+

TLC PROFILE: The ethyl acetate extract from the leaves of *Orthosiphon stamineus* were loaded on to pre coated TLC (60 F_2 54) and it was developed using solvent system in the ratio of Petroleum ether, Chloroform and methanol (1:0.5:0.1, V/V/V) was used for the development of the exudates on silica gel plates silica gel 60 F_{254} (10x20 cm, 0.2mmlayer). Visible and the non-visible spot given and it is fluorescent with UV light at 360nm and 240nm. The Rf value of compounds were shown in Fig-1.



TLC plate viewed under 240 nm of UV light

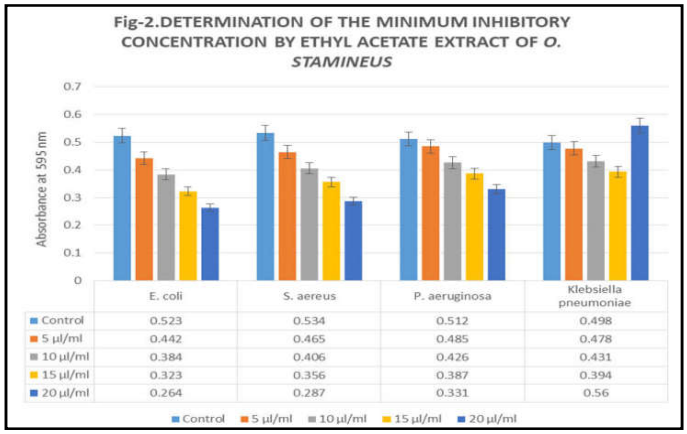
TLC plate viewed under 360 nm of UV light

ANTIBACTERIAL ACTIVITY BY DISC DIFFUSION METHOD: Antibacterial activity of ethyl acetate extract from the leaves of *Orthosiphon stamineus* tested against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were assessed as inhibition zones in the agar plates (Table-2). In this experimental all the bacteria were found to be sensitive to the ethyl acetate extract. Additionally, the zone of inhibition reconsideration that the ethyl acetate extract influenced antibacterial activity in proportion to concentration gradient ranges 25-100 µl/ml against the tested bacteria. Amongst the bacteria considered, *Escherichia coli* (17.8 mm) and *Staphylococcus aureus* (16.3 mm) were recognized to be highly susceptible followed by *Pseudomonas aeruginosa* (13.7 mm) and *Klebsiella pneumonia* (12.3 mm). This may confirm the antibacterial property of ethyl acetate extract of *O. stamineus*. Previously, Ho *et al*., (2014) O. stamineus contained rich in phenolic metabolites, and also have an in vitro study using a disc diffusion assay showed that the extracts of this plant have antimicrobial activity against the selected foodborne bacteria. *The inhibitory Zone size measured included the 6.0 mm size of the well by means of caliper. All the assays were duplicated, and the mean values were recorded.

Table-2. The antibacterial activity of the ethyl acetate extract from the leaves of *O. stamineus* by disc diffusion method

Pathogenic organism	Different concentrations Crude extract (µl/ml)			
	25 µl/ml	50 µl/ml	75 µl/ml	100 µl/ ml
Escherichia coli	10.2±0.5	12.8±2.1	15.9±1.2	17.8±0.4
Staphylococcus aureus	9.2±1.2	11.7±0.4	13.8±0.8	16.3±1.7
Pseudomonas aeruginosa	7.5±0.8	9.6±2.7	11.7±0.1	13.7±0.9
Klebsiella pneumoniae	6.7±0.5	7.6±1.3	9.4±0.8	12.3±1.6

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC): Tested of ethyl acetate extract of O. stamineus showed antimicrobial activity against the bacterial strains by MIC Fig-2. Based on the result, it was found that the ethyl acetate extract of O. stamineus strongly inhibited the growth of Escherichia coli, Staphylococcus aureus. The most resistant strains among Gram-positive bacteria turned out to be Staphylococcus aureus. In the case of Gram-negative bacteria, it was found that ethyl acetate extract of O. stamineus strongly inhibited the growth of Escherichia coli. Pseudomonas aeruginosa turned out to be the most resistant strains of Gram-negative bacteria.



ABTS RADICAL ASSAY: The antiradical activity of ethyl acetate extract of *O. stamineus*, as the demonstrative of nutritional food source, were evaluated in vitro by ABTS assay, as well as by assessment of possible to discoloration of ABTS. Substantial changes were witnessed for the ethyl acetate extract of *O. stamineus*, as well as between the assays employed. In table-3 the outcomes of antioxidant activity gained for tested samples, as well as Vitamin-C used as standard are presented. According to previous reports, polyphenols and flavonoids in *herbal plants* exhibit a variety of bioactivities, especially antioxidant properties. Therefore, with different approaches and mechanisms, the four most common antioxidant activity assays (Li *et al*., 2008). The mechanism of action on antioxidants can help to neutralize reactive oxygen species generated in human body, reducing tissue damage and alleviating oxidative stress (Ge and Ma, 2013).

Table-3. Free radical-scavenging ability using ABTS assay of ethyl acetate extract of *O. stamineus*

Different concentration of extract	ABTS radical activity	
	Ethyl acetate extract of *O. stamineus*	Standard Vitamin-C
25 µl/ml	17.32±0.28	16.32±2.89
50 µl/ml	33.65±1.89	30.21±0.23
75 µl/ml	47.31±2.36	43.21±2.69
100 µl/ml	66.23±1.47	62.34±1.78
EC_{50} value	63.21	65.32

[a]Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

INHIBITION OF LIPID PEROXIDATION ACTIVITY: Inhibition of lipid peroxidation experimental was used as substrate egg yolk for free radical facilitated lipid peroxidation, which is a non-enzymatic method. Ethyl acetate extract of *O. stamineus* inhibited the lipid peroxidation brought by ferrous sulfate in egg yolk homogenates. Determined inhibition was documented in ethyl acetate extract of *O. stamineus* 81.23% with EC_{50} value 53.21 µl/ml and lowermost inhibition percentage ascorbic acid 76.32% with EC_{50} 58.64 *(Table-4)*. As it is recognized that lipid peroxidation is the remaining outcome of any free radical attack on membrane and other lipid components present in the system. Lipid peroxidation has been concerned to be a harmful element of many oxidative inflammatory disorders, in most cases lipid oxidation has not been observed as a connective step in the evolution of the disease. One exclusion to this is atherogenesis, where lipid oxidation has been recommended to be an automatous module of the formation of fatty streaks and atheroma (Steinberg *et al*., 1989).

Table 4. Inhibition of lipid peroxidation activity of ethyl acetate extract of *O. stamineus*

Different concentration of extract	Inhibition percentage of Lipid peroxidation	
	Ethyl acetate extract of *O. stamineus*	Standard Vitamin-C
25 µl/ml	20.31±1.49	17.32±2.36
50 µl/ml	44.32±0.98	41.23±1.78
75 µl/ml	65.23±1.56	57.32±1.36
100 µl/ml	81.23±2.36	76.32±1.49
EC_{50} value	53.21	58.64

[a]Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

NITRIC OXIDE RADICAL SCAVENGING ASSAY: Nitric oxide radical scavenging assay was carried out on the ethyl acetate extract of *O. stamineus* leaves from a concentration of 25 to 100 µg/mL. Percentage free radical scavenging was plotted against concentration of the extracts as shown in table-5. The ethyl acetate extract of *O. stamineus* displayed antioxidant activity through opposing with oxygen to scavenge for the nitrite radical which was generated from SNP at physiological pH in an aqueous environment. The radical activity increased with an increase in concentration of the ethyl acetate extract of *O. stamineus* reaching a flat terrain. Increasing the concentration of the extracts did not result in an increase in the nitrite radical scavenging activity. As antioxidants donate protons to the nitrite radical, the absorbance is decreased. The decrease in absorbance was used to measure the extent of nitrite radical scavenging (Rao *et al*., 2010). It was observed that all the extracts both ethanol and water scavenged nitrite radical in a dose dependent manner and reached a plateau in which a further increase in the concentration of the extracts did not result in an increase in the quenching of the nitrite radical (Nagmoti *et al*., 2011).

Table 5. Nitric oxide radical scavenging assay of the ethyl acetate extract of *O. stamineus*

Different concentration of extract	Percentage of Nitric oxide radical scavenging activity	
	Ethyl acetate extract of *O. stamineus*	Ascorbic acid (+ve control)
25 µl/ml	22.36±0.87	19.32±1.89
50 µl/ml	44.32±2.34	42.31±2.38
75 µl/ml	65.32±1.58	59.32±1.67
100 µl/ml	83.21±2.78	77.32±2.46
EC_{50} Value	49.32	53.21

[a]Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of three experiments.

CONCLUSION

In conclusion, the results obtained in the present study indicated that ethyl acetate extract of *O. stamineus* leaves antibacterial and potent antioxidants. Hence, *O. stamineus* leaves extracts can be used as natural sources of antibacterial and antioxidants as they could have great importance as therapeutic agents in preventing or slowing the progress of bacterial infection and aging related oxidative stress related degenerative diseases. *O. stamineus* leaves also have potential application in industry as natural antioxidants that could be used as food additives to prevent food deterioration.

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