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Antiradical and antibacterial effects of phenolic rich fraction from the leaves of *Orthosiphon stamineus*

M Durgadevi[1], K Prakash[2]
[1] Research Scholar, Department of Botany, Arignar Anna Government Arts College for Women, Walajapet, Tamil Nadu, India
[2] Assistant Professor, Department of Botany, Arignar Anna Government Arts College, Villupuram, Tamil Nadu, India

Abstract
The antioxidant and antibacterial properties of a Phenolic-rich *Orthosiphonstamineus leaf* extract were investigated in this study. The antibacterial activity was tested using the disc diffusion method and the determination of minimum inhibitory concentration, while the antioxidant activity was determined using reducing power activity, metal chelating activity, and inhibition of lipid peroxidation assay. It also had the highest metal chelating activity and suppression of lipid peroxidation (EC_{50} of μg mL^{-1}), as well as the highest reducing power ($A_{700} = 0.462$) at 100 μg mL^{-1}. Against both Gram-negative and Gram-positive bacteria, it was found to have considerable antibacterial activity when the extracts were tested. These discoveries provided a scientific foundation for *Orthosiphon stamineus's* traditional use, as well as demonstrating its viability as a rich source of natural antioxidant and antibacterial compounds.

Keywords: *Orthosiphon stamineus***;** phenolic rich extract, antioxidant and antibacterial activity

Introduction
Herbs are an excellent source of metabolites that produce positive active chemicals. Because these organic bioactive substances have been shown to be capable of battling many human infections, further research into them would assist to fortify the medical system. More than 55 percent of the medications used in current pharmacopoeia are produced from herbs or from the modification of phytoconstituents (Stankovic *et al*., 2016) [6]. Modern testing have shown its usefulness in primary healthcare, particularly among populations in less industrialised or developing countries (80 percent). It is anticipated that in the future, 75–90% of the population would rely on herbal medicines. This bodes well for tribal and traditional medicine, since 85 percent of treatments are plant-based (Johnson and Ayoola, 2015) [5]. The phytochemicals claimed to have a complex pharmacokinetic profile, which entails establishing the time course of phytoconstituents, as well as their absorption, circulation, metabolism, and excretion. This pharmacokinetic profile aids in comprehending the complex relationship between the intensity and time sequence of pharmacology and the toxicological consequences of phytochemicals in humans. Furthermore, it allows the expansion of prospects for its use and recognition by various regulatory agencies. There is insufficient data on the chemical compositions, pharmacodynamics, pharmacokinetics, and metabolomics of traditional complementary medicinal herbs, according to the WHO.
Plants, particularly fruits and vegetables, are high in natural antioxidants. Many antioxidant substances found naturally in plants have been identified as free radical scavengers (Al-Rifai *et al.*, 2017) [7]. Currently, several scientists are looking for naturally occurring antioxidants to replace synthetic antioxidants such as butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA), which are suspected of causing carcinogenic effects in foods, medical materials, and pharmaceutical items (Chouhan and Singh, 2011) [1]. Furthermore, natural antioxidants have the power to improve food quality and stability, as well as operate as nutraceuticals in biological systems to terminate free radical chain reactions (Djeridane *et al*., 2006) [2].
Reactive oxygen species (ROS) and reactive nitrogen species (RNS) caused by oxygen and nitrogen radicals, as well as other free radical-mediated reactions, are involved in ageing (Biswas *et al.*, 2011), neurological disorders, cancer, atherosclerosis, gastric ulcer, and other ailments. According to several epidemiological research, persons who eat a diet rich in fresh fruits and vegetables have a lower risk of cardiovascular and other degenerative diseases (Rababah *et al.*, 2011) [3]. Antioxidant activities of natural substances have been the subject of several studies (Tsuruga *et al*., 2007) [8].

Materials and Methods

Plant Collection

Orthosiphon stamineus found in K.K. Nagar's fresh vegetable market was collected (June 2019). Dr. S. Sankaranarayanan, Head, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai-600 106, Tamilnadu (Voucher specimen NO. MB-456/22GSMC), authenticated the plant (Voucher specimen NO. MB-456/22GSMC).

Phenolic Rich Fraction

Orthosiphon stamineus was prepared by crushing 100 g of dried plant in a blender to a paste-like state for 1 minute. First, the homogenised sample was freeze dried to minimise moisture content for a more efficient extraction process. The powder was then defatted by soaking it in n-Hexane for 24 hours. Then it was steeped in methanol for 72 hours to yield methanol crude extract, that was concentrated using a rotatory evaporator (40 °C). To obtain chloroform soluble fractions, the sticky residues were partitioned with chloroform. It was evaporated at a low pressure and dried with ethyl acetate (Dasgupta *et al.,* 2014).

Phytochemical Screening

Standard phytochemical screening procedures were used to evaluate the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins, and polyphenols in *Orthosiphon stamineus* aqueous extract (Harborne 1973; Trease and Evans 1983).

Reducing Power Activity

According to Yen and Chen [37], the total reducing power was determined using potassium ferricyanide as a reagent. *O. stamineus* phenolic rich fraction was combined with an equal amount of 0.2 M phosphate buffer, pH 6.6, and 10 mg g-1 potassium ferricyanide and incubated for 20 minutes at 50° C. The mixture was further centrifuged at 6000×g for 10 minutes with an equal volume of 10 mg g-1 trichloroacetic acid added. In a 1:1:2 ratio, with a part of the upper layer, distilled water and 1 mg g−1 $FeCl_3$ were mixed. At 700 nm, the absorbance was measured. Increased reaction mixture absorbance indicated increased reducing power.

Metal Chelating Activity

According to Iihami *et al*., the metal chelating capacity of the phenolic rich fraction of O. stamineus was measured (2003). 1 ml of various flavonoid-rich fraction concentrations were added to 0.05 ml of 2mM ferric chloride solution. The reaction was started by adding 0.2 ml of 5 mM Ferrozine to the mixture and rapidly shaking it. The absorbance was measured at 562 nm against blank after 10 minutes. Ascorbic acid was employed as a standard and all measurements were taken in triplicate. The following equation was used to obtain the percent inhibition of the ferrozoine-Fe2+complex.

% Inhibition of ferrozoine-Fe2+complex = $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was theabsorbance of flavonoid rich fraction.

Inhibition of Lipid Peroxidation Activity

The approach of Ohkawa *et al*. (1979) was used to analyse lipid peroxidation generated by the Fe^{2+} ascarbate system in egg yolk as thiobarbituric acid reactive substances (TBARS). In a final volume of 0.5 ml, the experimental mixture contained 0.1 ml of egg yolk (25 percent w/v) in Tris-HCl buffer and various concentrations of phenolic rich fraction of *O. stamineus*. For 1 hour, the experimental combination was incubated at 37°C. Following the incubation period, 0.4 ml of the sample was taken and treated with 0.2 ml sodium dodecyl sulphate (1.1%), 1.5 ml thiobarbituric acid (0.8%), and 1.5 ml acetic acid (20 percent, pH3.5). With distilled water, the final amount was increased to 4.0 ml, which was then held in a water bath at 95 to 100 °C for 1 hour. After cooling, the reaction mixture was added with 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v), shaken rapidly, and centrifuged at 4000 rpm for 10 minutes. To measure TBARS, the absorbance of the butanol-pyridine layer was measured at 532 nm in a Deep Vision (1371) UV–Vis Spectrophotometer. By comparing the optical density (OD) of the test sample to that of the control, the inhibition of lipid peroxidation was evaluated. The standard was ascorbic acid.

The percentage inhibition of lipid peroxidation by each extract was determined using 1-(E/C) × 100,

Where C is the absorbance of the totally oxidised control and E is the absorbance of the test sample.

Antibacterial Activity

The disc diffusion method, developed by Bajalan *et al.,* (2017) [9] was used to test the antibacterial activity of crude alkaloid extracts. Bacteria were cultured overnight on Mueller Hinton agar plates, and five colonies were suspended in 5 ml of sterile saline (0.9 percent) with a bacterial population of $3x10^8$ CFU/ml. To remove the extra fluid, a sterile cotton swab was dipped into the solution and swirled several times with gentle pressure on the inside wall of the tube. The swab was used to inoculate the dried surface of the MH agar plate streaking four

times, rotating the plate roughly 90º to ensure a uniform dispersion of the inoculums. Before adding a sterile disc with a diameter of 9 mm, the medium was allowed to dry for around 3 minutes. To ensure uniform contact with the bacteria, each disc was firmly placed on the agar. Weighing and dissolving the bioactive component (50 g) in 1 cc of 7% ethyl acetate. The inhibition zone was measured and estimated after 24 hours of incubation at 37°C. The experiments were repeated three times in total. By measuring the zones of growth inhibition surrounding the discs, the results (mean value, n=3) were recorded.

Minimum Inhibitory Concentrations

The isolated compounds' minimal inhibitory concentrations were established using the dilution method (Baczek *et al.*, 2017) [10]. The strains were raised to exponential phase in Mueller Hinton broth with an A560 of 0.8, corresponding to 3.2×10^8 CFU/ml. The phenolic rich fraction of *O. stamineus* was diluted to produce solutions with concentrations of 25, 50, 75, and 100 µg/ml. In separate test tubes containing 4ml of MHbroth injected with 0.5 ml bacterial suspension at a final concentration of 10^6 CFU/ml, 0.5 ml of each concentration was added. Five independent tests were conducted in duplicate to ascertain each MIC. The bacterial control tubes included 4.5 ml of bacterial inoculates and 0.5 ml of 7 percent acetone, while the blank tubes had 4.5 ml of uninoculated MH broth and 0.5 ml PBS. The tubes were incubated at 37°C for 18 hours, and the absorbance at 560 nm was used to measure bacterial growth inhibition.

Statistical Analysis

All of the tests were carried out in triplicate. The data was analysed using the SPSS software version 20. The findings of three trials are provided as mean values with standard deviation (SD). The experimental data was evaluated using one-way analysis of variance (ANOVA) for multiple comparisons, and Duncan's test was utilised when the results were significant.

Result and Discussion

Phytochemical Screening

The phytochemical screening of *O. stamineus* aqueous extract revealed the presence of alkaloids, flavonoids, polyphenols, and terpenoids, but not glycosides or tannins (Table -1).

Table 1: Phytochemical screening of aqueous extract of *O. stamineus*

Sl. No.	Phytochemical Constituents	Observation	Phenolic rich fraction of *O. 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	-

+ indicate positive result; -- Indicate negative result

Reducing Power Activity

The reducing power of different concentrations of O. *stamineus* phenolic rich fraction was tested, and it was found to be concentration dependant (Graph-1). Positive control and cooked extract have lower reducing power than the phenolic rich fraction of *O. stamineus*. Antioxidant activity and reducing power are known to be linked. The presence of reductants, which have been shown to have antioxidative potential by breaking the free radical chain and donating a hydrogen atom, determines a sprout's reducing ability. The reduction of the Fe^{3+}/ferricynide complex to the ferrous form is caused by the presence of deductants in the phenolic rich fraction of *O. stamineus*. As a result, the production of Perl's Purssian blue at 700 nm can be used to monitor Fe^{2+} levels.

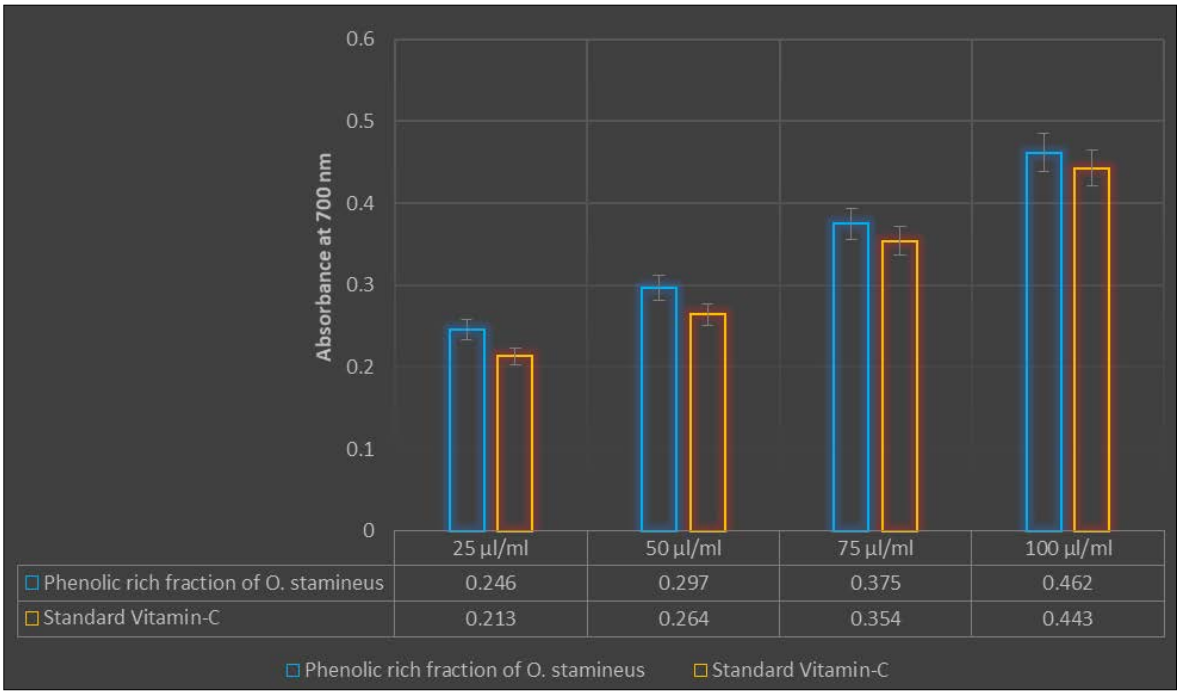

Fig 1: Reducing power activity of phenolic rich fraction from the leaves of *O. Stamineus*

Metal Chelating Activity

Table-2 shows the metal chelating properties of the phenolic rich fraction of O. *stamineus*. The ability of phenolic-rich fractions of *O. stamineus* to compete with ferrozine for ferrous iron in solution was tested. The phenolic rich fraction of *O. stamine*us caused the development of ferrous and ferrozine complexes in this study, indicating that it has chelating activity and can capture ferrous iron before ferrozine. The phenolic rich fraction of *O. stamineus* instantly reduced the greenish blue colour complex and had the maximum chelating activity (67.32 percent) when compared to the positive control.

Table 2: Metal chelating activityof phenolic rich fraction of *O. stamineus*

Different concentration of extract	Metal chelating of phenolic rich fraction of *O. stamineus*	Standard Vitamin-C
25 µl/ml	15.34±0.87	13.64±0.78
50 µl/ml	35.32±0.89	27.32±0.26
75 µl/ml	48.32±1.56	45.32±1.78
100 µl/ml	67.32±2.34	64.32±2.34
EC_{50} Value	71.32±0.28	77.32±1.58

[a]In comparison to the control, the results are expressed as a percentage of Metal chelating activity. The mean+SD of three experiments is shown by each value.

Inhibition of Lipid Peroxidation Activity

In this non-enzymatic assay, egg yolk was utilised as a substrate for free radical driven lipid peroxidation. Normally, phenolic compounds' antioxidant action is due to their ability to neutralise lipid free radicals and prevent the breakdown of hydroperoxides into free radicals. This produces MDA and other aldehydes, which combine with TBA to form a pink chromogen that absorbs at 532 nm. As indicated in Table-3, the phenolic rich fraction of *O. stamineus* had a 78.32 percent lipid peroxidation inhibitory impact, whereas Vitamin-C had a 73.32 percent lipid peroxidation inhibitory effect. The findings were concentration-dependent and statistically significant.

Table 3: Inhibition of lipid peroxidation activity by phenolic rich fraction of *O. stamineus*

Different concentration of extract	Phenolic rich fraction of *O. stamineus*	Standard Vitamin-C
25 µl/ml	20.31±1.46	16.34±2.34
50 µl/ml	33.64±0.89	29.32±2.78
75 µl/ml	57.34±2.39	45.31±1.39
100 µl/ml	78.32±2.34	73.32±2.45
EC_{50} value	54.21±1.34	59.32±2.34

[a] The results are expressed as a percentage of lipid peroxidation inhibition compared to the control. The mean+SD of three experiments is shown by each value.

Effect of phenolic rich fraction of *O. Stamineus* on the growth of pathogenic bacteria

Antibacterial activity of *O. stamineus* phenolic rich fraction was tested against Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, and Pseudomonas aeruginosa as inhibition zones in agar plates (Table-4). The phenolic rich fraction of *O. stamineus* was found to be sensitive to all of the microorganisms examined. Furthermore, the zone of inhibition investigation demonstrated that the flavonoid-rich fraction had antibacterial activity against the tested microorganisms in response to concentration gradient ranges of 25-100 µl/ml. Staphylococcus aureus (18.3mm) (Gram positive) and Escherichia coli (16.7mm) were shown to be particularly sensitive among the microorganisms examined, followed by Pseudomonas aeruginosa and Enterococcus faecalis. This could explain the antibacterial properties of *O. stamineus'* phenolic rich fraction.

Table 4: The antibacterial activity of the phenolic rich fraction 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**
Staphylococcus aureus	10.2±1.7	13.6±1.4	16.3±1.4	18.3±3.2
Pseudomonas aeruginosa	8.5±1.3	10.3±1.4	13.6±0.3	15.7±2.3
Escherichia coli	9.3±2.7	11.4±1.8	14.3±1.7	16.7±1.6
Enterococcus faecalis	7.3±1.3	9.3±1.7	10.9±1.6	14.2±2.3

*Using a calliper, the inhibitory Zone size was measured, which included the well's 6.0 mm diameter. The mean values of all the assays were reported after they were duplicated.

Analysis of polyphenolic rich fraction by GC–MS

The largest flavonoid content was found in the phenolic rich fraction of *O. stamineus* leaves, which also had the strongest antiradical and antibacterial activity. The chemical makeup that may contribute to its activity is determined via GC-MS analysis. The GC–MS study revealed a number of different phenolic chemicals (Fig-2 and Table-5). Furthermore, several cinnamic acid derivatives with the phenolic hydroxyl group were regarded to be antiradical and antibacterial, and their high free radical scavenging properties were considered to offer a variety of health benefits.

Table 5: Analysis of phenolic rich fraction of *O. stamineus* leaves by GC–MS

S.No	Compound	Retention Time(min)	Molecular weight	Major peaks
1	Ledene oxide	16.87	167	251, 219, 201, 167
2	Pentadecanoic acid, 14-methyl-, methyl ester	17.2	270	269, 226, 184
3	3-Methylene-4-phenyltricyclo	17.8	224	251, 209, 180
4	Tricyclo[7.2.2.0(3,8)]tridec-12-en-2-one, 5,6-epoxy-4-methyl	18.35	218	251, 227, 209
5	Tricyclododeccarboxyethoxy	19.65	273	259, 241, 222
6	*Benzamide, 2-amino-5-hydroxy*	18.9	228	259, 227, 209
7	Eicosatetraenoic acid	20.48	304	253, 238, 224
8	Hexadec-9-enoic acid	22.37	254	255, 218, 194
9	Tetratriacontane	22.67	478	308, 294, 266
10	8-Octadecenal	25.03	266	251, 218, 178
11	Heptacosane	25.53	380	379, 336, 280
12	Ethanol 2-(octadecyloxy)-	26.03	314	255, 223, 195
13	17-Pentatriacontane	29.35	506	318, 293, 251
14	Spathulenol	16.87	220	251, 219, 201

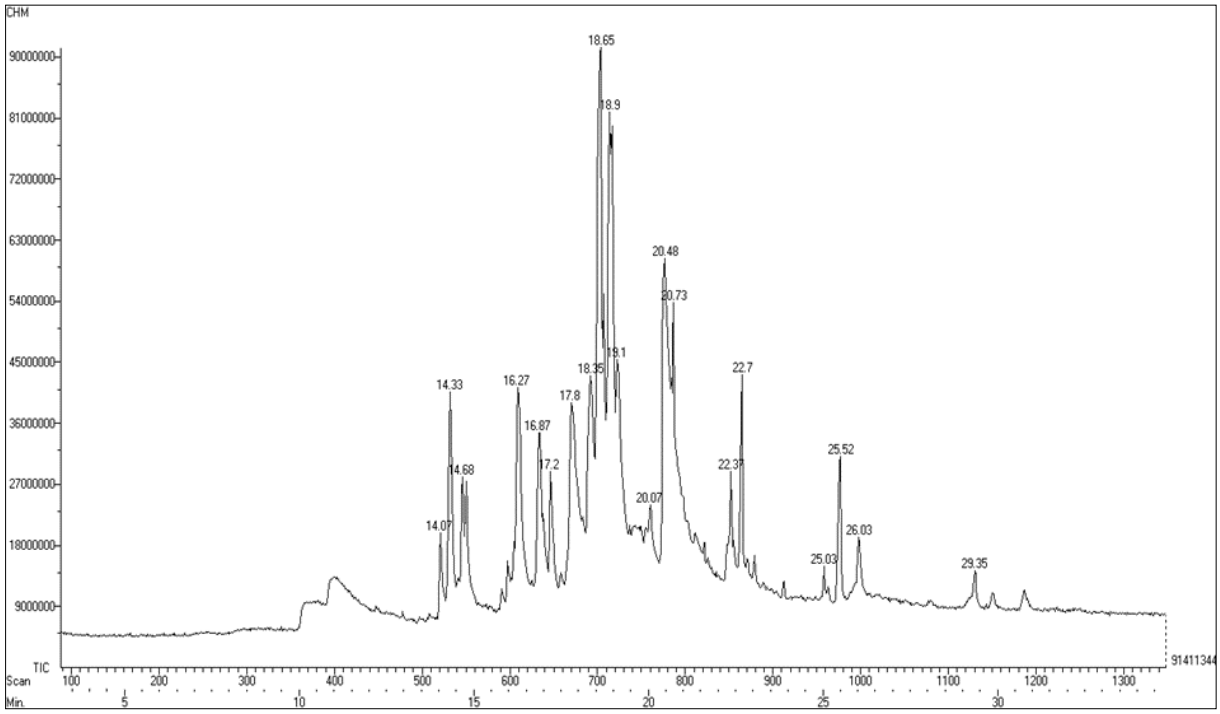

Fig 2: Analysis of phenolic rich fraction of *O. stamineus* leaves by GC–MS Consolidated retention Time

Conclusion

In an *In vitro* model, a phenolic-rich fraction of *O. stamineus* displays potential antiradical and antibacterial activity. The phenolic rich fraction of *O. stamineus* was scientifically verified by the results of an *In vitro* investigation. Furthermore, the phenolic rich fraction of *O. stamineus* includes active biomarkers that may be responsible for antiradical and antibacterial activity. The total antioxidant capacity of *O. stamineus's* phenolic rich fraction is given as μg of ascorbic acid activity equivalent per μg of extract.

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