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Antioxidant and antibacterial activity of polyphenol rich fraction form leaves of *Ocimum gratissimum*: Siddha traditional medicinal plants

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Abstract
To determine the antioxidant and antibacterial abilities of the polyphenol rich fraction form leaves of *Ocimum gratissimum*. The total polyphenol content was evaluated using Folin-Ciocalteu assay. ABTS, lipid peroxidation, superoxide and nitric oxide scavenging capacity assays were performed to test the antioxidant activity. Subsequently, the antimicrobial activities against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* bacterial strains were assessed using disc diffusion methods. Results of polyphenol rich fraction form leaves of *Ocimum gratissimum* contained high total polyphenol content. The polyphenol rich fraction form leaves of *Ocimum gratissimum* showed effective antioxidant capacity EC_{50} value of ABTS, lipid peroxidation 54.23 and 65.45µg/mL respectively. Additionally, the flavonoid rich fraction was effective against both Gram-positive bacteria. In conclusion flavonoid rich fraction from leaves of *O. gratissimum* demonstrated valuable antioxidant and antimicrobial properties.

Keywords: antioxidant; antibacterial; *Ocimum gratissimum;* polyphenol

Introduction

Plants and other natural products are still in great demand due to various factors like their safety, dependability and lesser side effects. World Health Organization has made an attempt to identify all medicinal plants used globally and listed more than 20,000 species. Most of the medicinal plant parts are used as raw drugs and they possess varied medicinal properties (Shantabi et al., 2008). Plants have a great potential for producing new drugs and used in traditional medicine to treat chronic and even infectious diseases. The Phytomedicine are more import in the treatment of inflammation. In recent years, there is an increasing awareness about the importance of medicinal plants. Many medicinal plants have shown to exhibit potent antioxidant and anti-inflammatory effect in the treatment of inflammation by using various models (Tilburt and Kaptchuk, 2008) [16].

The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists and food manufacturers as consumers move toward functional foods with specific health effects. The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes (Tan et al., 2016). Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Moreover, knowledge and application of such potential antioxidant activities in reducing oxidative stresses *in vivo* has prompted many investigators to search for potent and cost-effective antioxidants from various plant sources (Suttirak and Manurakchinakorn, 2014) [14].

Flavonoids are ubiquitous in photosynthesising cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases. Increasingly, this class of natural products is becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids possessing antifungal, antiviral and antibacterial activity. Moreover, several groups have demonstrated synergy between active flavonoids as well as between flavonoids and existing chemotherapeutics. Reports of activity in the field of antibacterial flavonoid research are widely conflicting, probably owing to inter- and intra-assay variation in susceptibility testing.

Ocimum gratissimum, popularly known as basil, basil-clove, or alfavaca, serves as a condiment in human food and is also widely known and used for its therapeutic properties. Previous studies reported anesthesia, anti-stress, antidiarrheal, anthelminthic, antiinflammatory, antimutagenic, antipyretic, antiulcerative, gastroprotective, fungicidal, hepatoprotective. It is used in Siddha and Ayurveda traditional medicine for treating digestive system disorders, such as stomach ache and diarrhoea, kidney complaints, and infections. The leaves are used for treating whooping cough and various types of fever. A leaf decoction is used for treating coughs in India. The leaves are rubbed between the palms and sniffed as a treatment for blocked nostrils, they are also used for abdominal pains, sore eyes, ear infections, coughs, barrenness, fever, convulsions, and tooth gargle, regulation of menstruation and as a cure for prolapse of the rectum (Matasyoh et al., 2007) [10].

In the coastal areas in is used in the treatment of epilepsy, high fever and diarrhoea. In the Savannah areas decoctions of the leaves are used to treat mental illness. The flowers and the leaves of this plant are rich in essential oils so it is used in preparation of teas and infusion (Cristiana et al., 2006) [4].

Material and Methods

Plant material

Ocimum gratissimum was obtained from Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai, Tamil Nadu, India. A plant taxonomist authenticated the plant and samples were kept in the Medicinal Botany herbarium with voucher specimen numbers MB/GSMC-354/2021. The leaves were sufficiently air-dried in 5 days at the ambient room temperature, while the flower was cut into smaller pieces and air-dried in 7 days.

Phytochemical screening

The aqueous extract of *Ocimum gratissimum* leaves 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 (Aida et al., 2001; Hess et al., 1995) [1, 7].

Total phenol and flavonoid content

The total phenolic content was determined using Folin-Ciocalteu reagent according to the method of Singleton et al. (1999) [13]. A total amount of 40 µL crude methanol extract (1 mg/mL) was mixed with 200 µL of Folin-Ciocalteu reagent and 1160 µL of distilled water. After 3 min, 600 µL of 20% sodium carbonate (Na_2CO_3) was added. The mixture was shaken for 2 h at room temperature, and absorbance was measured at 765 nm. All tests were performed in triplicate. Gallic acid was used as a standard for the calibration curve. The concentration of total phenolic compounds was expressed as µg of gallic acid equivalents per 1 mg of extract using the following equation obtained from a standard gallic acid graph.

Estimation of flavanoid

A 1ml aliquot of each aqueous extract of *Ocimum gratissimum* leaves was mixed thoroughly with 1ml of 2% aluminium chloride and 0.5 ml 0f 33% acetic acid followed by the addition of 90% methanol and the content is thoroughly stirred and allowed to stand for 30 minutes (Elfalleh et al., 2019) [6]. The absorbance was measured at 414 nm using a UV-Visible Spectrophotometer. Quercetin was used as a standard.

Extraction of Polyphenols

Polyphenols were extracted from crushed leaves of *Ocimum gratissimum* (100 g), according to the method of Zhang et al. (2000).

The fraction was completed twice at 20 °C in a shaking incubator. Methanol/acetone/water (3.5:3.5:3, v/v/v) containing 1 % formic acid were used extracting solvents were 100 mL at 30 min. The extract was then filtration through Whatman No.1 filter paper. The filtrates solution were evaporated under vacuum at 40 °C to remove methanol and acetone.

Lipophilic colours materials were removed from the aqueous phase by two consecutive extractions in a separator funnel with a twofold volume of petroleum ether. The aqueous phase was finally collected and further extracted three times by ethyl acetate (ethyl acetate: aqueous phase = 1:1, *v/v*) in the separator funnel. The ethyl acetate phases were collected, evaporated and dried under vacuum at 35 °C to obtain polyphenol sample.

ABTS *(*2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging assay

ABTS radical scavenging activity of polyphenol rich fraction form leaves of *O. gratissimum* was followed by Re et al. (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

$$\text{ABTS Scavenging Effect (\%)} = [(A_0 - A_1/A_0) \times 100]$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of polyphenol rich fraction form leaves of *O. gratissimum*.

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 Badmus et al. (2010). 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 leaves of *Ocimum gratissimum* 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.

Superoxide radical scavenging assay

This assay was based on the capacity of the polyphenol rich fraction form leaves of *O. gratissimum* to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution:

$$\% \text{ Super oxide radical scavenging capacit y} = [(A_0\text{-}A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance sample.

Nitric oxide radical scavenging activity

Nitric oxide scavenging ability of polyphenol rich fraction form leaves of *O. gratissimum* was measured according to the method described by Makhija et al. (2011). 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of extracts and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation:

$$\% \text{ Nitric oxide radical scavenging capacity} = [(A_0\text{-}A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of polyphenol rich fraction form leaves of *O. gratissimum*.

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 ~3×10^8 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 polyphenol rich fraction form leaves of *O. gratissimum* 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.

Statistical analysis

All the experiments were done in triplicate. The SPSS software version 20 was used for data analysis. The results are expressed as mean values with standard deviation (±SD) from three experiments. The experimental data obtained were analyzed for multiple comparisons using one-way analysis of variance (ANOVA) and when the results were significant, Duncan's test was also used.

Result and Discusion

Phytochemical screening

The phytochemical screening of aqueous leaf extract of *O. gratissimum* studied presently showed the presence of alkaloids, flavonoids, polyphenol, terpenoids, and absence of glycosides and tannin (Table-1). Plant secondary metabolites such as tannins, saponins, flavonoids, alkaloids and other plant metabolites compounds are serve as protection against predation by many microorganism include insects and other herbivores (Kaur and Kapoor, 2002) [9].

Table 1: Phytochemical screening of aqueous leaf extract of *O. gratissimum*

Sl. No.	Phytochemical Constituents	Observation	Aqueous leaf extract of *O. gratissimum*
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

Total phenolic and flavonoid content

In this study, the initial experiments exposed that aqueous leaf extract of *O. gratissimum* 60 °C for 60 min since it afforded a maximum yield of phenolics. The total phenolic content of the aqueous fresh extract, calculated from the calibration curve (R^2 = 0.998), was 53.64 ±2.45 gallic acid equivalents/g, and the total flavonoid content (R^2 = 0.999) was 41.23±1.89 rutin equivalents/g (Table-2).

ABTS radical assay

The antiradical activity of polyphenol rich fraction form leaf of *O. gratissimum*, 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 polyphenol rich fraction, 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. It can be evidently understood that polyphenol rich fraction exhibited prominent antioxidant activity, expressively higher than Vitamin-C. Nevertheless, in current experimental presented that these activities were mainly due to presence of polyphenol compounds. The $ABTS^{\bullet+}$ chromophore is produced through the reaction between ABTS and potassium persulfate which converts ABTS into its radical cation. The $ABTS^{\bullet+}$ is reactive towards most antioxidants including phenols (Walker and Everette, 2009) [17].

Table 2: Free radical-scavenging ability using ABTS assay of polyphenol rich fraction form leaf of *O. gratissimum*

Different concentration of extract	ABTS radical activity	
	Polyphenol rich fraction form leaf of *O. gratissimum*	Standard Vitamin-C
25 µl/ml	22.31±0.89	17.34±0.79
50 µl/ml	39.65±3.45	33.64±2.78
75 µl/ml	58.32±2.36	55.34±2.34
100 µl/ml	79.32±1.45	72.31±1.56
EC_{50} value	54.23	59.32

[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. Polyphenol rich fraction form leaves of *O. gratissimum* inhibited the lipid peroxidation brought by ferrous sulfate in egg yolk homogenates. Determined inhibition was documented in polyphenol rich fraction form leaves of *O. gratissimum* 68.32% with EC_{50} value 65.45 µl/ml and lowermost inhibition percentage ascorbic acid 63.21% with EC_{50} 69.31 µl/ml (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, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). The collaboration of oxygen with certain molecules leads to the formation of free radicals and once formed, the chief danger comes from the damage they can cause when they counter with important cellular mechanisms including DNA, proteins and the cell membrane (Droge, 2002) [5].

Table 3: Inhibition of lipid peroxidation activity of polyphenol rich fraction form leaves of *O. gratissimum*

Different concentration of extract	Inhibition percentage of Lipid peroxidation	
	Polyphenol rich fraction form leaves of *O. gratissimum*	Standard Vitamin-C
25 µl/ml	19.32±1.89	17.34±0.89
50 µl/ml	34.56±2.36	32.57±2.64
75 µl/ml	49.32±1.89	44.32±1.63
100 µl/ml	68.32±2.45	63.21±1.57
EC_{50} value	65.45	69.31

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

Superoxide scavenging activity

Polyphenol rich fraction form leaves of *O. gratissimum* displayed authoritative scavenging activity for superoxide radicals in a concentration dependent development than positive control. Polyphenol rich fraction form leaves of *O. gratissimum* exhibited maximum radical activity in the percentage of 74.32% with EC_{50} value 56.32 µl/ml when related to positive control 69.56% with EC_{50} Value 63.21 µl/ml (Table-5). One of the typical process to produce Superoxide radicals is concluded photochemical decrease of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. The result obviously specifies that the polyphenol compound has an evident outcome as superoxide radical scavenging properties. Other plant extracts and certain plant flavonoids including mangiferin, naringin, quercetin, myricetin and rutin have been found to scavenge superoxide free radical in a concentration-dependent manner (Jagetia and Venkatesha, 2005) [8].

Table 4: Superoxide scavenging activity of polyphenol rich fraction form leaves of *O. gratissimum*

Different concentration of extract	Percentage of Superoxide scavenging activity	
	Polyphenol rich fraction form leaves of *O. gratissimum*	Standard Vitamin-C
25 µl/ml	17.32±0.89	15.34±0.87
50 µl/ml	31.23±2.34	27.32±1.56
75 µl/ml	53.32±1.45	47.36±1.46
100 µl/ml	74.32±1.36	69.56±1.63
EC_{50} value	56.32	63.21

[a] Results are expressed as percentage of Superoxide scavenging activity with respect to control. Each value represents the mean+SD of three

Nitric oxide radical scavenging

Polyphenol rich fraction form leaves of *O. gratissimum* indicated a strong nitric oxide scavenging ability which was equivalent to the standards ascorbic acid. The EC_{50} value (51.23 µl/ml) of polyphenol rich fraction form leaves of *O. gratissimum* was less than ascorbic acid (54.31 µl/ml). Percentage of nitric oxide radical scavenging activity polyphenol rich fraction form leaves of *O. gratissimum* and standards were presented in Table-7. In the present outcome, nitrite was formed by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by polyphenol rich fraction. Imperative scavenging activity may be due to the antioxidant property of polyphenol, compounds present in leaves of *O. gratissimum,* which contest with oxygen to respond with nitric oxide, prominent to less production of nitric oxide.

Table 5: Nitric oxide radical scavenging assay of polyphenol rich fraction form leaves of *O. gratissimum*

Different concentration of extract	Percentage of Nitric oxide radical scavenging activity	
	Polyphenol rich fraction form leaves of *O. gratissimum*	Standard Vitamin-C
25 µl/ml	23.64±1.78	19.32±0.46
50 µl/ml	39.64±2.89	36.54±1.56
75 µl/ml	57.32±1.46	54.32±1.89
100 µl/ml	83.21±2.36	76.32±2.73
EC_{50} value	51.23	54.31

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

Antibacterial activity

Antibacterial activity of polyphenol rich fraction form leaves of *O. gratissimum* tested against *Enterococcus faecal is*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* were assessed as inhibition zones in the agar plates (Table-8). In this experimental all the bacteria were found to be sensitive to the polyphenol rich fraction. Additionally, the zone of inhibition reconsideration that the polyphenol rich fraction influenced antibacterial activity in proportion to concentration gradient ranges 25-100 µl/ml against the tested bacteria. Amongst the bacteria considered, *Staphylococcus aureus* and *Escherichia coli* was identified to be highly susceptible followed by *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Polyphenols metabolites such as phenolic and flavonoids, are significant antibacterial activity (Clementi *et al.*, 2014) [3]. Moreover, it has been reported that large number of different chemical compounds such as (phenolic compounds and its derivative compounds) are presented in organic solvent extracts of plants, and thus these chemical components can affect multiple target sites against the bacterial cells (Burt, 2004) [2].

Table 6: The antibacterial activity of the polyphenol rich fraction form leaves of *O. gratissimum* 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	9.5±0.2	11.8±1.2	14.5±0.4	16.3±1.3
Pseudomonas aeruginosa	7.4±2.5	9.8±1.5	12.1±1.3	14.6±0.5
Escherichia coli	8.7±1.3	10.6±0.8	13.7±1.6	15.7±1.8
Enterococcus faecalis	7.1±0.9	9.5±2.4	11.6±1.4	13.4±2.3

[*]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.

Conclusions

The present study verified that polyphenol rich fraction form leaves of *O. gratissimum* were found to contain a high amount of total phenolic and total flavonoids. Likewise, the extracts revealed significant antioxidant activities against various tests: ABTS, Lipid peroxidation superoxide and Nitric scavenging activity. Furthermore, polyphenol rich fraction form leaves of *O. gratissimum* antibacterial activity against various human pathogens and indicate that it might be valuable in treating infectious diseases caused by microorganisms. In general, the results prove the effectiveness of the plant for its potent antioxidant and antimicrobial activities. Accordingly, the positive values of the plant regarding its application in traditional medicine have been confirmed.

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