

RESEARCH ARTICLE

PHYTOCHEMICAL INVESTIGATION AND STUDY ON ANTIOXIDANT PROPERTIES OF
OCIMUM CANUM HYDRO-ALCOHOLIC LEAF EXTRACTS*Behera Saiprasanna¹, Babu S Manohar², Ramani Y Roja³, Choudhury Prasanta K¹, Panigrahi Rajeshree¹¹Department of Pharmacology, Royal College of Pharmacy and Health Sciences, Berhampur, Odisha- 760002²Department of Pharmacology, SIMS College of Pharmacy, Mangaldas Nagar, Guntur- 522001³Department of Pharmacology, MKCG Medical College, Berhampur, Odisha- 760004*Corresponding author's Email: behera.saiprasanna82@gmail.com, saiprasanna_behera@rediffmail.com, Phone: 09861252518

ABSTRACT

Many herbal remedies individually or in combination have been recommended in various medical expositions for the cure of different diseases. Free radicals are implicated for many diseases including diabetes mellitus, arthritis, cancer, aging, etc. In the treatment of these diseases, antioxidant therapy has gained utmost importance. Currently there has been an increased interest globally to identify antioxidant compound that are pharmacologically potent and have low or no side effects. As plants are source of natural antioxidants, much attention has been gain to plants. The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenges over the last two decades. A variety of free radical scavenging antioxidants exists within the body in which many of them are derived from dietary sources like fruits, vegetables and teas. The *Ocimumcanum*, commonly known as 'Kala Tulsi', has been recognized in different system of traditional medicines for the treatment of different diseases and ailments of human beings. In this study, Antioxidant activity of the hydro-alcoholic extract of *Ocimumcanum* was determined by various antioxidant assays. In all the testing, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals. These findings suggest that the hydro-alcoholic extracts are able to scavenge free radicals, by either hydrogen or electron donating mechanisms, and can therefore act as primary antioxidants. The antioxidant property may be related to the phenols and flavonoids present in the extracts. These results clearly indicate that *Ocimumcanum* can be effectively used against free radical mediated diseases.

Key words: *Ocimumcanum*, phytochemical constituents, traditional uses and pharmacological properties, antioxidant activity

INTRODUCTION

Natural products are important sources for biologically active drugs¹. There has been an increasing interest in the medicinal plants as natural products in different parts of the world². Medicinal plants containing high antioxidant properties play an important role in the prevention of various degenerative diseases in the society. The medicinal value of these plants depends on bioactive phytochemical constituent's action in the human body. Some of the most important bioactive phytochemical constituents include alkaloids, flavonoids, essential oils, tannins and saponins³. Phenolics are commonly found in medicinal plants and their biological effects, include antioxidant activity. Due to synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), and tert-butyl hydroquinone (TBHQ), which are widely used in food industry and cosmetic, have been growing concern over the possible carcinogenic effects⁴. Thus interest in natural antioxidant has increased considerably. Nowadays, it is well known that natural antioxidants extracted from herbs and spices have high antioxidant properties and are used in many food applications⁵. Natural antioxidants from plant sources are potent and safe due to their harmless nature; wild herbs have their antioxidant properties⁶. Various plants are proposed to be antioxidants as their essential oils contain phenylpropanoids in high contents. Essential oils of plant genus *Ocimum* belonged to family Lamiaceae. It is collectively called as Basil, is a diverse and rich source of aromatic essential oil. The species such as *Ocimumbasilicum* L., *O canum* Sims., *O gratissimum* L. and *Ocimum sanctum* L. are composed of interesting phenyl-propenes e.g. eugenol, methyl eugenol and methyl

chavicol. Essential oils of these plants have been broadly used as culinary herbs, as pharmaceutical agents because of their antimicrobial, antiemetic, antidiabetic, antifertility, antiasthmatic, antistress and anticancer activity⁷.



Figure 1: *Ocimumcanum*- Wild basil, from Yamasaki Lab. Plant Photo Gallery, by kind permission from Kazuo Yamasaki

Ocimum canum Sims (Hairy Basil) is a traditional medicinal plant distributes throughout Odisha and it is commonly known as KalaTulasi in Odisha has an unusual mint-like flavor. The plant branches out from its base, with angle stems and open foliage. The plant shows a pungent, aromatic flavor and is commonly cultivated for culinary

purposes. *O. canum* is used specially for treating various types of diseases and lowering blood glucose and also treats cold, fever, parasitic infestations on the body and inflammation of joints and headaches⁸. Essential oil from the leaves of *O. canum* possesses antibacterial and insecticidal properties⁹. It is used in ritual as an incense as well to protect the home and welcome newborns into the world. It is an unusual and very useful addition to the medicinal garden. The hairy leaves and decorative flowers are very aromatic and form a lush mound about 2 feet in height. This annual plant grows well in full sun, well-drained soil and plenty of heat. The plant branches from the base and has an angled stems and oval pubescent leaves. Its leaves are tiny and fuzzy and have violet or white flowers, having a sweet scent resembling that of the clove. The leaves of the *Ocimum canum* are opposite and toothed. It is irregular and occurs in crowded whorls. The *Ocimum canum* has a small corolla. These plants have intense floral-fruity aromas. The oil of the *Ocimum canum* is composed of Linalool. The seeds may provide fiber or reduce constipation.

MATERIALS AND METHODS

2.1. Plant collection

Leaves of *Ocimum canum* were collected in the month of December 2011 from its natural habitat from nearby Mohuda village, Berhampur, Ganjam district of Odisha. The plant was authenticated from Department of Botany, Khalikote College, Berhampur, Odisha. The leaves were cleaned and dried under the shade to avoid degradation of volatile oil. The leaves were dried in hot air oven at 55°C for 3 days and at 40°C for the next 4 days

2.2. Extraction

The dried leaves were coarsely powdered and extracted with a mixture of methanol: water (7:3, v/v) by a Soxhlet apparatus at 50°C. The solvent was completely removed and obtained dried crude extract which was used for investigation. Further the extracts were subjected for the phytochemical study as well as pharmacological screening.

2.3. Phytochemical screening

Phytochemical screenings were performed using standard procedures.^{10,11,12}

1) **Test for alkaloids:** To the extract dilute hydrochloric acid will be added and filtered. The filtrate will be treated with various alkaloidal reagents

a) **Mayer's test:** The filtrate will be treated with Mayer's reagent: appearance of cream colour indicates the presence of alkaloids.

b) **Dragendroff's test:** The filtrate will be treated with Dragendroff's reagent: appearance of reddish brown precipitate indicates the presence of alkaloids.

c) **Hager's test:** The filtrate when treated with Hager's reagent, appearance of yellow colour precipitate indicates the presence of alkaloids.

2) Test for carbohydrates and reducing sugar:

The small quantities of the filtrate will be dissolved in 4ml of distilled water and filtered. The filtrate will be subjected to

a) **Molisch's test:** A small portion of the filtrate will be treated with Molisch's reagent and sulphuric acid. Formation of a violet ring indicates the presence of carbohydrates.

b) **Fehling's test:** The extract will be treated with Fehling's reagent A and B. The appearance of reddish brown colour precipitate indicates the presence of reducing sugar.

c) **Benedict's test:** The extract will be treated with Benedict's reagent; appearance of reddish orange colour precipitate indicates the presence of reducing sugar.

d) **Barfoed's test:** The extract will be treated with barfoed's reagent and heated. Appearance of reddish orange colour precipitate indicates the presence of non-reducing sugars.

3) Test for steroids:

Libermannburchard's test: The extract will be treated with 3ml of acetic anhydride, few drops of glacial acetic acid followed by a drop of concentrated sulphuric acid. Appearance of bluish green colour indicates the presence of steroids.

4) Test for proteins:

a) **Biuret test:** The extract will be treated with copper sulphate solution, followed by addition of sodium hydroxide solution; appearance of violet colour indicates the presence of proteins.

b) **Millon's test:** The extract will be treated with Millon's reagent; appearance of pink colour indicates the presence of proteins.

5) Test for tannins:

The extract will be treated with 10% lead acetate solution; appearance of white precipitate indicates the presence of tannins.

6) Test for phenolic compounds:

a) The extract will be treated with neutral ferric chloride solution; appearance of violet colour indicates the presence of phenolic compounds.

b) The extract will be treated with 10% sodium chloride solution; appearance of cream colour indicates the presence of phenolic compounds.

7) Test for flavonoids:

a) 5ml of extract will be hydrolyzed with 10% sulphuric acid and cooled. Then, it will be extracted with diethyl ether and divided into three portions in three separate test tubes. 1ml of diluted sodium carbonate, 1ml of 0.1N sodium hydroxide, and 1ml of strong ammonia solution will be added to the first, second and third test tubes respectively. In each test tube, development of yellow colour demonstrated the presence of flavonoids.

b) **Shinoda's test:** The extract will be dissolved in alcohol, to which few magnesium turnings will be added followed by concentrated HCL drop wise and heated, and appearance of magenta colour shows the presence of flavonoids.

8) Test for gums and mucilage:

The extract was treated with 25 ml of absolute alcohol, and filtered. The filtrate will examine for its swelling properties.

9) Test for glycosides:

When a pinch the extract was treated with glacial acetic acid and few drops of ferric chloride solution, followed by the addition of conc. Sulphuric acid, formation of ring at the junction of two liquids indicates the presence of glycosides.

10) Test for saponins:

Foam test About 1 ml of the extract was diluted to 20 ml of with distilled water and shaken well in a test tube. The formation of foam in the upper part of test tube indicates the presence of saponins.

11) Test for Triterpenoids:

The substance was warmed with tin and thionyl chloride. Pink colour indicates the presence of triterpenoids.

2.4. Determination of antioxidant activity**a) DPPH radical scavenging assay¹³**

To the Methanol solution of DPPH (1 mM) an equal volume of the extract dissolved in alcohol was added at various concentrations from 250 to 2000 µg/ml in a final volume of 1.0 ml. An equal amount of alcohol was added to the control. After 20 min, absorbance was recorded at 517 nm. Experiment was performed in triplicate.

b) ABTS radical scavenging assay¹³

To the reaction mixture containing 0.3 ml of ABTS radical, 1.7 ml phosphate buffer and 0.5 ml extract was added at various concentrations from 250 to 2000 µg/ml. Blank was carried out without drug. Absorbance was recorded at 734 nm. Experiment was performed in triplicate.

c) Nitric oxide radical scavenging¹³

Sodium nitroprusside (5µM) in standard phosphate buffer solution was incubated with different concentration of the test extracts dissolved in standard phosphate buffer (0.025M, pH 7.4) and the tubes were incubated at 25 °C for 5 hr. After 5 h, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthylethylene di-amine dihydrochloride in water). The absorbance of chromophore formed was read at 546 nm. The control experiment was also carried out in similar manner, using distilled water in the place of extracts. The experiment was performed (in triplicate) and % scavenging activity was calculated using the formula:-

$$100 - [100/\text{blank absorbance} \times \text{sample absorbance}]$$

The activity was compared with ascorbic acid, which was used as a standard antioxidant.

d) Super oxide scavenging (NBT reduction assay)¹³

Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the test compound, 1 ml of alkaline DMSO and 0.2 ml of NBT 20

mM in phosphate buffer pH 7.4 was added. The experiment was performed in triplicate.

e) Iron chelating activity assay¹³

The reaction mixture containing 1 ml O-Phenanthroline, 2 ml Ferric chloride, and 2 ml extract at various concentrations ranging from 250 to 2000 µg/ml in a final volume of 5 ml was incubated for 10 minutes at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of extract and absorbance obtained was taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without extract. Experiment was performed in triplicate.

f) Hydroxyl Radical Scavenging Activity (Deoxyribose degradation assay)

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al.*, (1987)¹⁴. Stock solutions of EDTA (1mM), FeCl₃ (10 mM), ascorbic acid (1mM), H₂O₂ (10mM) and deoxyribose (10mM) were prepared in distilled deionized water. The assay was performed by adding 0.1 ml of EDTA 0.01 ml of FeCl₃, 0.1 ml of H₂O₂, 0.36 ml of deoxyribose, 1.0 ml of plant extract (250-2000 µg/ml), 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1hr. About 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA to develop the pink chromogen, measured at 532 nm. The hydroxyl radicals scavenging activity was calculated using the following equation:-

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

Where, A₀ was the absorbance of the control (blank) and A₁ was the absorbance in the presence different concentrations of the extract

g) Total reduction capability¹⁵

The Fe³⁺ reducing power of the extract was determined by the method of Oyaizu *et al.*, with a slight modification. Different concentrations (250–2000 µg/mL) of extract (0.5 mL) were mixed with 0.5-mL Phosphate buffer (pH 6.6) and 0.5-mL 0.1% potassium hex-cyanoferrate, followed by incubation at 50°C in water bath for 20 min. After incubation, 0.5-mL 10% TCA was added to terminate the reaction. The upper portion of the solution (1 mL) was mixed with 1 mL of distilled water and 0.1 mL 0.01% FeCl₃ solution was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against appropriate blank solution. All tests were performed three times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

h) Scavenging Activity of Hydrogen Peroxide¹⁶

This activity was determined according to a methodsuggested by B. Halliwell and groupwith minor changes. Aliquot of 50-mM H₂O₂ and various concentrations (0–2000 µg/mL) of samples were mixed (1: 1 v /v) and incubated for 30 minutes at room temperature. After incubation, 90µl of the H₂O₂-sample solution was mixed with 10 µl HPLC-grade methanol and 0.9-mL FOX reagent was added (*previously prepared by mixing 9*

volumes of 4.4-mM Ascorbic acid in HPLC -grade methanol with 1 volume of 1-mM xylenol orange and 2.56-mM ammonium ferrous sulfate in 0.25-M H₂SO₄). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of ferric-xylenol orange complex was measured at 560 nm. All tests were carried out three times and sodium pyruvate was used as the reference compound

2.5 Statistical analysis¹⁷

The experimental results were expressed as mean ± SEM of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) and two way analysis of variance (ANOVA). All these analysis was done by Graph Pad Prism Software program (version 5). P values < 0.05 were regarded as significant.

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening

Phytochemical screening of the extracts revealed the presence of carbohydrates, flavonoids and tannins

3.2 Antioxidant assays

DPPH radical scavenging assay

The proton radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity.

The DPPH test provides information on their activity of the test compounds with a stable free radical. This assay determines the scavenging of stable radical species of DPPH by antioxidants. The degree of reduction in absorbance measurement by *Ocimumcanumis* indicative of the radical scavenging (antioxidant) power of the plant. The study showed that the hydro-alcoholic extract have the proton-donating ability and can serve as free radical inhibitors or scavenger, acting possibly as primary antioxidant.

Table 1:- Phytochemical analysis of *Ocimumcanum* leaves

S. No	Phytoconstituents	Ethanol	Aqueous
1	Alkaloids	-	-
2	Carbohydrates	+	+
3	Glycosides	-	-
4	Phytosterols	-	-
5	Fixed oils	-	-
6	Saponins	-	-
7	Tannins	+	-
8	Protein and amino acids	-	-
9	Gums and mucilage	-	-
10	Flavonoids	+	+
11	Terpenoids	+	+

+ = presence, - = absence.

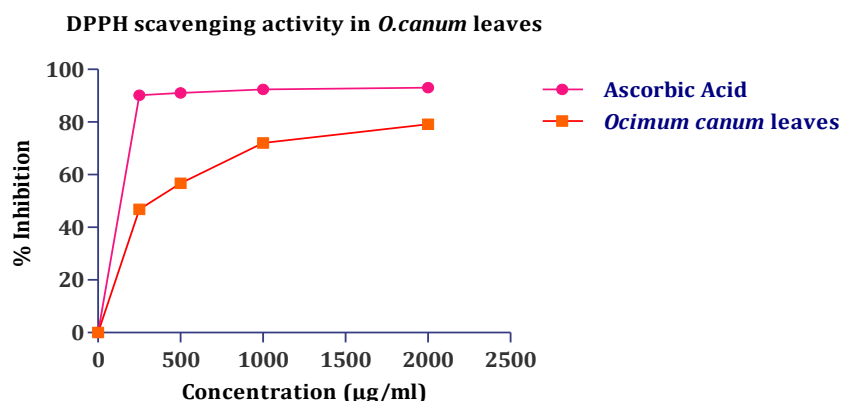


Figure 2: Study on DPPH scavenging activity in *Ocimumcanum* leaves at 517 nm

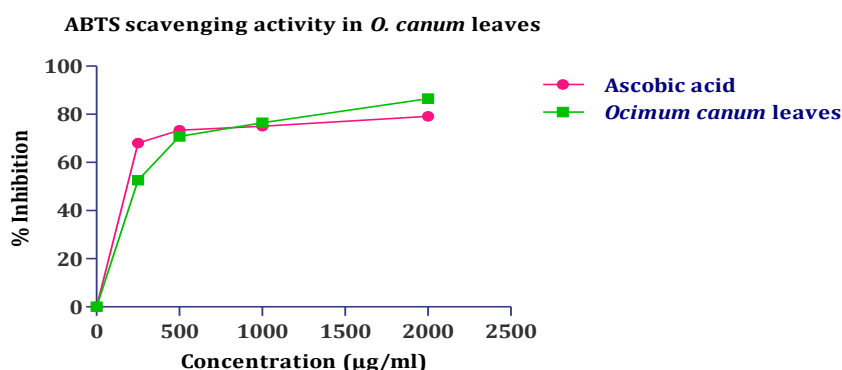


Figure 3: Study on ABTS scavenging activity in *Ocimumcanum* leaves at 405 nm

ABTS radical scavenging activity

ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals (Mathew and Abraham 2006). The

scavenging of the ABTS+ radical by the *O. canum* was found to be much higher than that of DPPH radical.

Nitric oxide scavenging

Sodium nitroprusside serves as a chief source of free radicals. The absorbance of the chromophore formed

during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthyl-ethylene-diamine is used as the marker for NO scavenging activity. *O. canum* leaf

extract possess significant free radical scavenging action against nitric oxide (NO) induced release of free radicals at the concentrations between 250-2000 µg/ ml.

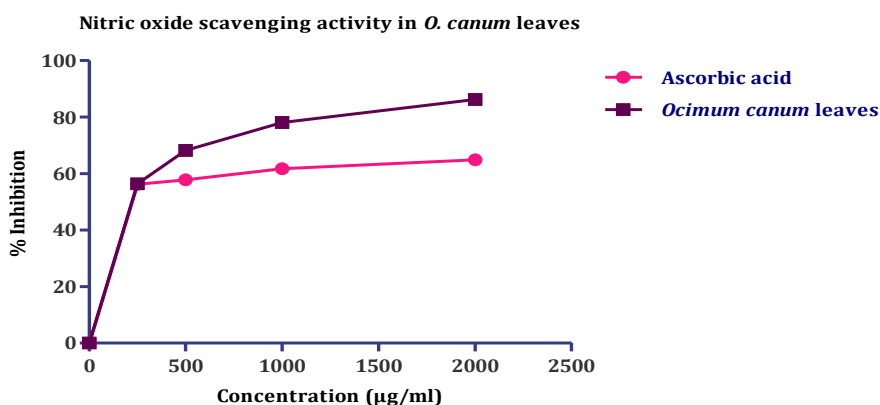


Figure 4: Study on Nitric oxide scavenging activity in *Ocimumcanum*leaves at 546 nm

Superoxide radical scavenging activity

Superoxide radical scavenging activity exhibited by the hydro-alcoholic extract of *Ocimumcanum*at different concentration showed that the superoxide scavenging

activity of *Ocimumcanum*was less as compared to the standard .The probable mechanism may be due to the non-inhibitory effect of hydro-alcoholic extract of the leaf towards generation of super oxides in the reaction mixture.

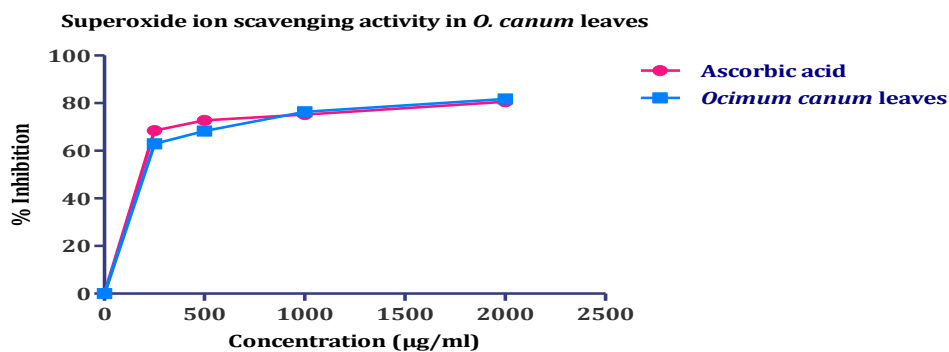


Figure 5: Study on Superoxide ion scavenging activity in *Ocimumcanum*leaves at 560 nm

Iron chelating activity assay

O-phenanthroline quantitatively forms complexes with Fe²⁺ which get disrupted in the presence of chelating agents. The hydro-alcoholic extract of

*Ocimumcanum*interferedwith the formation of a ferrous-o-phenanthroline complex, thereby suggesting that the extracts had metal chelating activity. This study suggests that *Ocimumcanum*possess antioxidant activity.

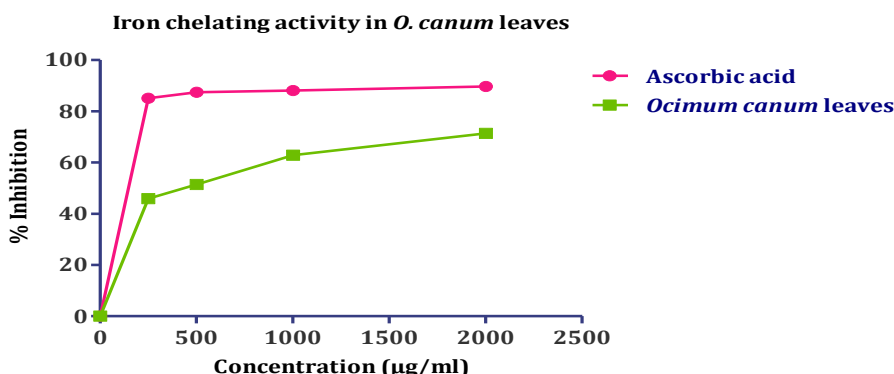


Figure 6: Study on Iron chelating activity in *Ocimumcanum* leaves at 510 nm

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity is measured as the percentage inhibition of hydroxyl radicals generated in the Fenton's reaction mixtureby studying the competition between deoxyribose and the extract for hydrogen radicals

generated from Fe²⁺/ascorbate/EDTA/H₂O₂systems. The hydroxyl radicals attack deoxyribose which eventually results in TBARS formation. From the present results, it is observed that the leaf extract of *O. canum*have better hydroxyl radical scavenging activity as reflected in terms of percentage inhibition

Hydroxyl Radical Scavenging Activity in *Ocimum canum* leaves

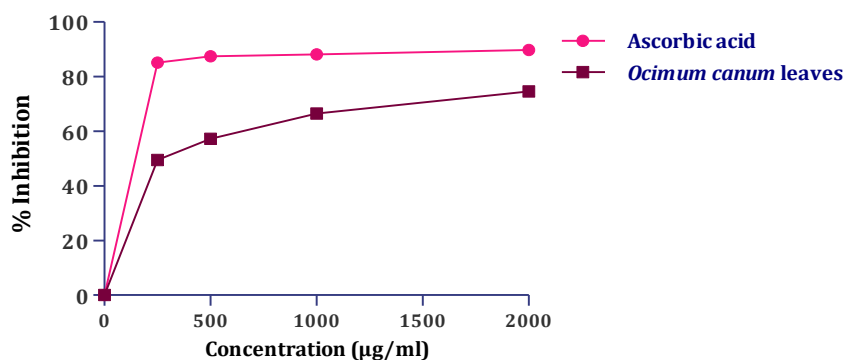


Figure 7: Study on Hydroxyl Radical Scavenging Activity in *Ocimumcanum* leaves

Total reduction capability in *Ocimum canum* leaves

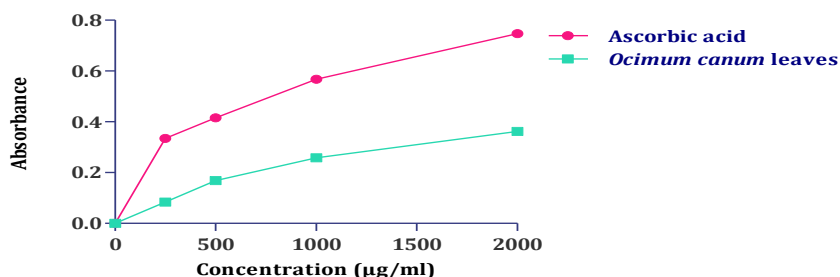


Figure 8: Study on Total reduction capability in *Ocimumcanum* leaves at 700 nm

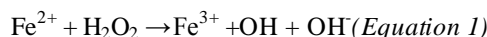
Total reduction capability¹⁸

Reducing power assay measures the electron-donating capacity of an antioxidant. The reduction of the ferric ion (Fe³⁺) to ferrous ion (Fe²⁺) is measured by the intensity of the resultant blue-green solution which absorbs at 700 nm, and an increased absorbance is indicative of higher reducing power. The reducing power of the extract increased progressively over the concentration range studied. Extract solutions at 1000µg/ ml had comparable reducing power to Ascorbic acid at 250 µg/ ml. These findings suggest that the *Ocimumcanum*hydro-alcoholic extract is capable of donating electrons, and could therefore react with free radicals or terminate chain reactions.

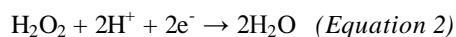
Scavenging Activity of Hydrogen Peroxide¹⁸

The scavenging of hydrogen peroxide by the standard (ascorbic acid) and extract after incubation for 10 minutes increased with increased concentration. *Ocimumcanum*hydro-alcoholic extracts

exhibited higher hydrogen peroxide scavenging activity than ascorbic acids at similar concentrations. While hydrogen peroxide itself is not very reactive, it can generate the highly reactive hydroxyl radical (OH) through the Fenton reaction (Equation 1). Thus, the scavenging of hydrogen peroxide is an important antioxidant defense mechanism.



The decomposition of hydrogen peroxide to water involves the transfer of electrons as in Equation 2.



The scavenging of hydrogen peroxide by phenolic compounds has been attributed to their electron-donating ability. The hydro-alcoholic OC extracts have high electron-donating abilities, and 68.21 ± 0.35 scavenging was achieved with concentrations of hydro-alcoholic extracts at 2000 µg/ ml. In comparison, the hydrogen peroxide scavenging activity of ascorbic acid at 2000 µg/ ml were found to be 98.79 ± 0.28

Scavenging Activity of Hydrogen Peroxide in *Ocimum canum* leaves

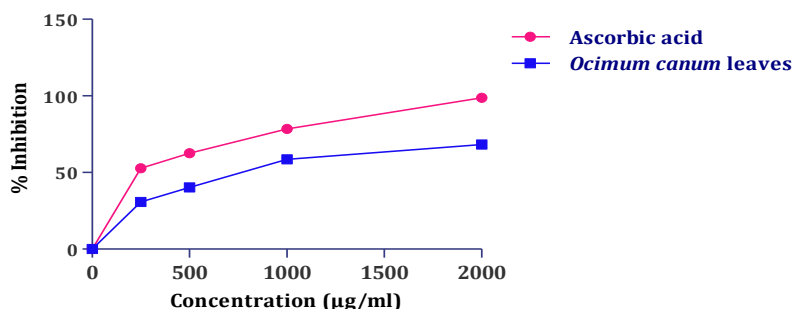


Figure 9: Study on Scavenging Activity of Hydrogen Peroxide in *Ocimumcanum* leaves at 560 nm

The present study clearly indicates that *Ocimumcanumis* a rich source of phyto-constituents having immense antioxidant potential. *Ocimumcanumis* rich in flavonoids, thus the results indicate that hydro-alcoholic leaf extract of *Ocimumcanum* have potent antioxidant activity, achieved by scavenging abilities observed against DPPH, ABTS, Nitric-oxide, Superoxide, Iron chelating, Hydroxyl radical, Reducing power assay and Hydrogen Peroxide.

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