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STUDIES ON ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL SCREENING OF SELECTED MEDICINAL PLANT *OCIMUM GRATISSIMUM* L.

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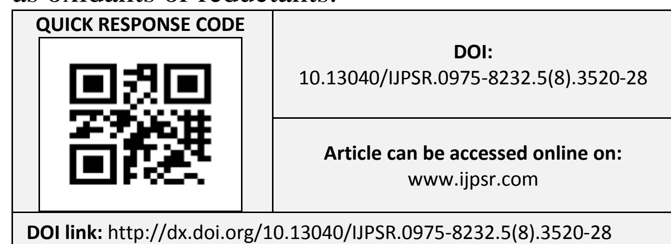
ABSTRACT: *Ocimum gratissimum* L. (O.G) has been used to treat various diseases since ancient time in India. This study investigated the antioxidant activity and phytochemical constituents in alcoholic extract of O.G. The alcoholic extract of O.G. was screened for antioxidant potential through DPPH assay and phytochemical analysis was undertaken through qualitative and quantitative methods. Different concentrations of extract were analyzed and ascorbic acid was used as a standard antioxidant. IC_{50} and % inhibition were evaluated. O.G. was found to have potent antioxidant ability of 82.40% at 200 µg/ml concentration and IC_{50} value at 37.92 µg/ml concentration. A good correlation was found to exist between concentration of extract and % inhibition with r^2=0.909. Phytochemical analysis revealed the presence of alkaloids, flavonoids, glycoside, tannin and phenolic compounds, triterpenoids and steroids, as well as 2.4 mg/g of photosynthetic pigments, 0.0003915 mg/g of ascorbic acid while 0.0910 mg/g of foliar phenol content was found to be present. These findings suggested that alcoholic extract of O.G. has potent antioxidant ability which could be the reason for its use in oxidative stress related diseases. The presence of phytochemicals like phenols and flavonoids in alcoholic extract could be responsible for this activity.

INTRODUCTION: A free radical can be defined as any molecular species capable of independent existence. Many radicals are unstable and highly reactive.

They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants.



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The most important oxygen-containing free radicals in many diseases are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. These are highly reactive species, capable of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids. Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body [1]. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions [1].

Oxidative stress has been postulated in many conditions, including atherosclerosis, inflammatory condition, certain cancers, and the process of aging [1].

The positive role of Vitamin E was found to be synergistic with ascorbic acid to ameliorate oxidative stress. In a study by Grases *et al* (2009), it was concluded that the antioxidant activity of herbal extracts could have an important role in avoidance of Calcium Oxalate Monohydrate papillary calculi formation [2, 3]. Thus, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke, neurodegenerative diseases and renal diseases etc. [4].

Although there are several enzymes system (superoxide dismutase, glutathione peroxidase and catalase) within the body that scavenges free radicals, the principle micronutrient (vitamins) which are non-enzymatic antioxidants, are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and β-carotene. The body cannot manufacture these micronutrients, so they must be supplied in the diet [1].

Despite the presence of the cell's antioxidant defense system to counteract oxidative damage from reactive oxygen species, oxidative damage accumulates during the life cycle, and radical-related damage has been proposed to play a key role in the development of age-dependent diseases [1].

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property [1].

A compound may exert antioxidant actions in vitro or in food by inhibiting generation of reactive species, or by directly scavenging them, or by raising the levels of endogenous antioxidant defences [5].

All the natural anti-oxidants though safer but show lower antioxidant activity than the synthetic anti-oxidants like BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoulene), but as these are considered to be promoters of carcinogenesis so need exists for safer, economic natural anti-oxidants with high anti-oxidant activity. The use of 1,1-diphenyl-2-picryl hydrazyl as a reagent for screening the antioxidant activity of small molecules and pure compounds or plant extracts has been reported [6].

The term "phytochemical" means naturally occurring plant chemicals or plant derived compounds that may affect health. They are secondary metabolites having a range of biochemical and physiological effects. Because many phytochemicals are not well characterized and their mode of action is not well established so research on these physiologically active plant components is currently an area of intense effort.

It includes alkaloids, carbohydrates, reducing sugar, flavonoids, glycoside, tannin & phenolic compound, saponin, proteins & amino acid, triterpenoids & steroids, photosynthetic pigments, foliar phenols and ascorbic acid etc. Various phytochemicals play an important role in disease prevention and treatment and promote health as well as they are considered responsible for conferring various disease preventing activities like antidiabetic, anticancer, antioxidant etc to the plants.

Ocimum gratissimum L. (O.G.) is a herbaceous perennial plant commonly known as scent leaf, found in tropical Asia especially India. *Ocimum gratissimum* L. *Var.* Clocimum is a new hybrid strain of O.G developed by Sobti *et al* from Indian Institute of Integrative Medicine formerly Regional Research Laboratory, Jammu Tawi. It has been used extensively in the traditional system of medicine in many countries.

It is used in the treatment of various diseases like cancer [7] antineoceptive, anti-inflammatory [8], anti-diahorreal [9], antibacterial [10], antifungal [11] and as nephroprotective [12].

Its ethanolic extract has shown various activities like analgesic [13] antifungal [14], aphrodisiac [15], hepatoprotective [16], antioxidant [17] and antidiabetic activity [18] etc. It has been reported to be rich in plant chemicals. The plant is known to contain

alkaloids, tannins, flavonoids, phenolic compounds and oligosaccharides [12, 19].

Various reports on phytochemicals present in ethanolic extract of O.G. showed the presence of carbohydrates, alkaloids, terpenoids, phenolics, tannins, flavonoids, anthraquinones, sterols, glycosides and saponins [20]. While the phytochemical analysis on both the fresh and dried leaves of the plant revealed the presence of terpenes, flavonoids, tannins, alkaloids, steroids, proteins, carbohydrates, fats and oils with the dried samples having higher concentrations [17].

The present study has been undertaken to evaluate the antioxidant activity through DPPH assay as well as qualitative phytochemical screening of ethanolic extract while quantitative phytochemical analysis of O.G. leaf was done.

MATERIALS AND METHODS:

Plant collection and identification: The plant of O.G. was collected from Kolar road, Bhopal, Madhya Pradesh, during the month of January 2013 and plant was identified with the help of regional Floras[21] and taxonomists and finally confirmed with the herbarium of Botanical Survey of India (BSI), Allahabad, voucher specimen No.90459.

Chemicals: All chemicals used were of high purity Merck grade. The compound 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich.

Preparation of plant extract: Fresh plant, after collection was shade dried at room temperature. Plant material was then grinded and the powdered plant material 100 g was extracted with alcohol by Soxhlet apparatus for 72 hours. Then the extract was concentrated in vacuo to dryness at 30-40°c temperature, obtaining dried extract which was stored in refrigerator until used for further analysis.

Experimental work

A) Measurement of antioxidant activity:

a. **DPPH radical scavenging assay:** The procedure for estimating the antioxidant activity involved UV-Spectrophotometric determination [22]. Three solutions i.e. standard, test and control were prepared.

Different solutions (25 – 100 µg/ml) of ascorbic acid were prepared in methanol. 1.5 ml of each solution of ascorbic acid were mixed with 1.5 ml of 200 µM DPPH solution and incubated for 30 min at room temperature in dark. Absorbance of each solution was taken against blank at 517 nm.

Different solutions of the given sample and control were prepared to give concentrations of 50 – 200 µg/ml. 1.5 ml of each solution of given sample was mixed with 200 µM DPPH solution and incubated at room temperature in dark. Absorbance of each solution of given sample was taken against blank at 517 nm.

Percentage of antioxidant activity of plant extract and Ascorbic acid was calculated by using formula:

$$I\% = \frac{Ac - (At - Ab)}{At} \times 100$$

Where, I% = percentage inhibition; Ac = absorbance of control (methanol and 200 µM DPPH solution); At = absorbance of ascorbic acid / given sample with DPPH solution; Ab =absorbance of ascorbic acid / given sample without DPPH solution.

B) **Phytochemical analysis:** Phytochemical testing was performed to assess the various phytoconstituents present in O.G. Qualitative analysis[23] of its ethanolic extract was performed to determine the presence or absence of carbohydrates, proteins and amino acids, glycosides, alkaloids, flavonoids, saponin, triterpenoids and steroids, tannin and phenolic compounds. While quantitative analysis was performed to determine the amount of chlorophyll, ascorbic acid as well as foliar phenols present in fresh plant material.

1. Qualitative analysis:

i. Tests for carbohydrates and reducing sugars:

Molisch test: 2 ml of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1 ml of concentrated sulphuric

acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Barfoed's test: 1 ml of extract and Barfoed's reagent were mixed in a test tube and heated on a water bath for 2 minutes. Red color due to formation of cupric oxide indicates the presence of monosaccharide.

Fehling's test: To 1 ml of aqueous extract, 1 ml of Fehling's A and 1 ml of Fehling's B solutions were added in a test tube and heated in a water bath for 10 minutes. Formation of red precipitate indicates the presence of reducing sugar.

Benedict's test: Equal volume of Benedict's reagent and extract were mixed in a test tube and heated in a water bath for 5-10 minutes. Solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution which indicates the presence of reducing sugar.

ii. **Tests for protein and amino acids:**

Ninhydrin test: 3 ml of the test solution was heated with 3 drops of 5% Ninhydrin solution in a water bath for 10 minutes. Formation of blue color indicates the presence of amino acids.

iii. **Tests for glycosides:**

Borntrager's test: To 3 ml of test solution, dilute sulphuric acid was added, boiled for 5 minutes and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and shaked thoroughly. The organic solvent layer was separated and ammonia was added to it. Formation of pink to red color in ammonical layer indicates the presence of anthraquinone glycosides.

Legal's test: 1 ml of test solution was dissolved in pyridine. 1 ml of sodium nitropruside solution was added and made alkaline using 10% sodium hydroxide solution. Formation of pink to blood red color indicates the presence of Cardiac glycosides.

Keller-Killiani test: To 2 ml of test solution, 3 ml of glacial acetic acid and 1 drop of 5% ferric chloride were added in a test tube. Then 0.5 ml of concentrated sulphuric acid was added carefully by the side wall of the test tube.

Formation of blue color in the acetic acid layer indicates the presence of Cardiac glycosides.

iv. **Tests for alkaloids:** To the extract, dilute hydrochloric acid was added, shaked and filtered. With the filtrate, the following tests were performed.

Mayer's test: To 2-3 ml of filtrate, few drops of Mayer's reagent were added along sides of the test tube. Formation of white or creamy precipitate indicates the presence of alkaloids.

Hager's test: To 1-2 ml of filtrate, few drops of Hager's reagent were added in a test tube. Formation of yellow color precipitate indicates the presence of alkaloids.

Wagner's test: To 1-2 ml of filtrate, few drops of Wagner's reagent were added in a test tube. Formation of reddish brown precipitate indicates the presence of alkaloids.

v. **Tests for flavonoids:**

Lead acetate test: The extract was treated with few drops of lead acetate solution. Formation of yellow precipitate may indicate the presence of flavonoids.

Alkaline reagent test: The extract was treated with few drops of sodium hydroxide separately in a test tube. Formation of intense yellow color, which becomes color less on addition of few drops of dilute acid, indicate the presence of flavonoids.

Shinoda test: To the extract, 5 ml (95%) of ethanol was added. The mixture was treated with few fragments of magnesium turning, followed by drop wise addition of concentrated hydrochloric acid. Formation of pink color indicates the presence of flavonoids.

vi. **Test for saponin:**

Foam test: The extract was diluted with distilled water and shaken in graduated cylinder for 15 minutes. The formation of layer of foam indicates the presence of saponins.

vii. **Tests for triterpenoids and steroids:**

Salkowski's test: The extract was treated with chloroform and then it was filtered. The filtrate was added with few drops of concentrated sulphuric acid, shaken and allowed to stand. If the lower layers turns red, sterol are present. Presence of golden yellow layer at bottom indicates the presence of triterpenes.

Liebermann-Burchard's test: The extract was treated with chloroform. To this solution few drops of acetic anhydride were added, boiled and cooled. Concentrated sulphuric acid was added through the sides of the test tube. A brown ring is formed at the junction of two layers, if upper layer turned green, indicates the presence of steroids and formation of deep red color indicates the presence of triterpenoids.

viii. **Tests for tannin and phenolic compounds:**

Ferric chloride test: Some amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added. Formation of blue, green or violet color indicates presence of phenolic compounds.

Lead acetate test: Some amount of extract was dissolved in distilled water. To this solution few drops of lead acetate solution was added. Formation of white precipitate indicates presence of phenolic compounds.

Dilute iodine solution test: To 2-3 ml of extract, few drops of dilute iodine solution were added. Formation of transient red color indicates the presence of phenolic compounds.

2. Quantitative analysis:

i. **Photosynthetic pigments content:** Photosynthetic pigments in O.G. leaf was analyzed by the method proposed by Machlachalan & Zalik (1963) and Duxbury & Yentsch (1956). Chlorophyll-a, Chlorophyll-b and carotenoids were determined by extracting the pigments from fresh leaf samples. Leaves were washed with distilled water and were cut into small pieces, 100 mg of these leaf pieces were put in 5 ml of 80% acetone and were crushed in a mortar pestle with little acid washed sand. The resultant extract was centrifuged at 5000 rpm for 5 minutes. Supernatant was collected and sediment was washed with 1 ml of 80% acetone and centrifuged again. The supernatant thus obtained is added to previous one and the total final volume of the supernatant was made upto 10 ml by adding 80% acetone. Optical density of the supernatant so obtained was recorded at 480, 510, 645 and 663 nm wavelengths, using Systronics Digital spectrophotometer Model-166, against blank carried out throughout the process.

The amounts of Chlorophyll-a, Chlorophyll-b and carotenoids were estimated in terms of mg/gm fresh weight of the leaf was calculated by using the following formulae (Machlachalan & Zalik, 1963; and Duxbury & Yentsch, 1956):

$$\text{Chlorophyll-a (mg/g fresh wt.)} = \frac{12.3\, D663 - 0.86\, D645}{d \times 1000 \times w} \times V$$

$$\text{Chlorophyll-b (mg/g fresh wt.)} = \frac{19.3\, D645 - 3.6\, D645}{d \times 1000 \times w} \times V$$

Total Chlorophyll= Chlorophyll-a + Chlorophyll-b (mg/g fresh wt.)

$$\text{Carotenoids (mg/g fresh wt.)} = \frac{7.6\, D480 - 1.49\, D510}{d \times 1000 \times w} \times V$$

Where V, is the volume of chlorophyll solution in acetone, d, is the light path, and w, is the fresh weight of plant part in g.

ii. **Ascorbic acid content:** Ascorbic acid content was estimated by using method proposed by Schaffert and Kingsley (1955). Reagents required for estimation were as follows: 4 % TCA (Trichloro acetic acid), 2 % 2-4 DNPH (2-4 Dinitro phenyl hydrazine), 85 % Sulphuric acid, 10 % Thiourea

Extraction of ascorbic acid: 2 g of plant material was crushed with 100 ml of 4 % TCA and then the contents were centrifuged at 5000 rpm. 20 ml of supernatant was taken and was mixed with ½ teaspoon of activated charcoal. This was shaken well and then filtered. From the filtrate, 4 ml filtrate was used for further analysis.

To 4 ml extract 1 drop of thiourea and 1 ml of 2-4 DNPH were added, the test tube containing sample was placed in a boiling water bath exactly for 10 minutes. After which the tubes were placed in a beaker containing crushed ice and 5 ml of 85% sulphuric acid was added to the sample slowly drop by drop and mixed by rotating the test tube placed in crushed ice. The sample was kept as such for 10 minutes and then optical density of this sample was read by using Systronics digital Spectrophotometer, Model-166 at 515 nm against blank carried throughout the process. The µg ascorbic acid content at a given optical density was determined with the help of a standard curve prepared by taking known amounts of ascorbic acid.

Standardization: 0.1 % solution of ascorbic acid was prepared by adding 100 mg of ascorbic acid in 100 ml of 4 % TCA. This served as the stock solution. From this stock solution, working ascorbic acid solution of 0.002 % concentration was prepared by mixing 0.02 ml of stock solution with 98 ml of 4% TCA. Aliquots of this working ascorbic acid solution were prepared as follows in separate test tubes. 0.1 ml, 0.15 ml, 0.2 ml, 0.25 ml, 0.3 ml, upto 1.0 ml. Each aliquot was diluted upto 4 ml by 4 % TCA. 1 drop of thiourea and 1 ml of 2-4 DNPH was added to each sample and the test tubes were placed in boiling waterbath for 10 minutes. Optical density of the samples was read at 515nm. From these optical densities and solutions of known concentrations, standard curve was plotted. With the help of standard curve ascorbic acid contents in samples were obtained. Total amount of ascorbic acid present in the sample was calculated by using the following formula:

$$\text{Total ascorbic acid} = \frac{\mu\text{g ascorbic acid X V}}{\text{W X 1000}}$$

Where, µg ascorbic acid = Concentration obtained from standard curve; V = Total volume of the sample; W= Weight of the plant sample.

iii. **Foliar phenol content:** Foliar phenols were estimated by the method proposed by Bray & Thorpe (1954). 200 mg of fresh plant material was homogenized with 10ml of 70% ethanol and centrifuged at 6000 rpm for 10 minutes. Residue was subjected to repeated washings with 70 % ethanol followed by centrifugation each time. Supernatant so obtained was concentrated by allowing evaporation overnight and by using a separating funnel the concentrate was partitioned through light petroleum to remove chlorophyll. To 1 ml extract in a test tube, 2 ml of 20% sodium carbonate solution and 1 ml 1N Folin Phenol reagent were added. The reaction mixture was immediately kept in boiling water bath exactly for 1 minute and cooled to room temperature before the optical density of this blue colored solution was measured at 650nm by using Systronics digital spectrophotometer, Model-166.

The µg phenol content at a given optical density was determined with the help of a standard curve prepared with known amount of quinine.

Total amount of phenol in the sample was obtained by using the following formula:

$$\text{Phenol content} = \frac{\mu\text{g phenol X V}}{\text{W X 1000}} \text{ (mg/g of fresh wt.)}$$

Where, µg phenol = concentration obtained from the standard curve; V= total volume of the mixture; W = weight of the sample.

RESULTS: In the present work, DPPH assay was utilized for evaluating the free radical scavenging action of O.G. In this assay, ascorbic acid was used as a standard compound and its IC_{50} value was determined to be 4.69 µg/ml (**Figure 1**).

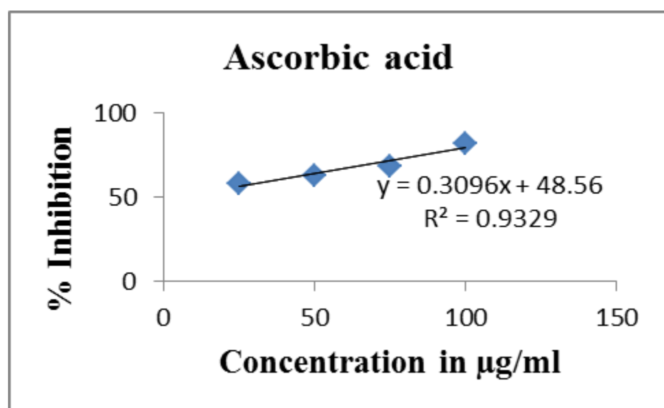

FIGURE 1: GRAPH REPRESENT REGRESSION CURVE OF ASCORBIC ACID BY DPPH ASSAY

Results show that, ethanolic extract of leaf of O.G. has potent antioxidant ability with an IC_{50} value at 37.92 µg/ml concentration and minimum % inhibition of 48.56% at 50 µg/ml and highest % inhibition was found to be 82.40% at 200 µg/ml concentration (**Figure 2**) which is comparable with that of ascorbic acid 82.05% at100 µg/ml. It was also found that a good correlation exists between the concentration of extract and % inhibition, with r^2 = 0.909 and the regression equation shows that the % inhibition is the dependent variable while concentration is independent and the % inhibition of DPPH free radical, increased with the increase in the concentration of extract.

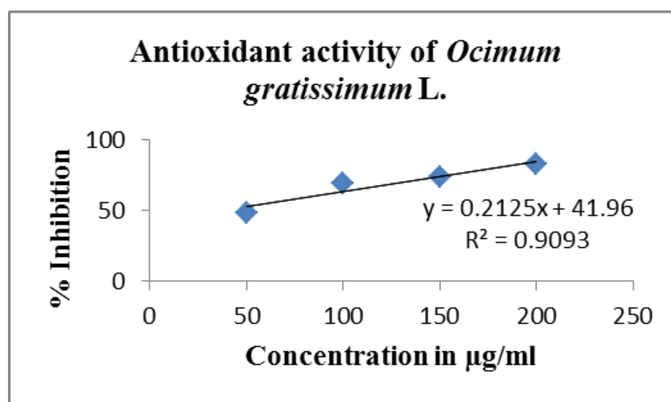

FIGURE 2: GRAPH REPRESENT REGRESSION CURVE OF *OCIMUM GRATISSIMUM* L. BY DPPH ASSAY

The results of qualitative phytochemical analysis of O.G. ethanolic extract revealed the presence of alkaloids, flavonoids, glycoside, tannin and phenolic compounds, triterpenoids and steroids (**Table 1**).

While quantitative phytochemical analysis showed the presence of 2.4 mg/g fresh weight of photosynthetic pigments, 0.0003915 mg/g fresh weight of ascorbic acid while 0.0910 mg/g fresh weight of foliar phenol content.

TABLE1: QUALITATIVE PHYTOCHEMICAL SCREENING OF *OCIMUM GRATISSIMUM* L. *EXTRACT*.

Phytochemical tested	Name of test	Observation
Carbohydrates and reducing sugars	Molisch's	-
Protein and amino acids	Barfoed's	-
	Fehling	-
	Benedict	-
	Ninhydrin	-
Glycoside	Borntrager	+
	Legal	+
	Keller- killiani	+
Alkaloids	Mayers	+
	Hagners	+
	Wagers	+
Flavonoids	Lead acetate	+
	Alkaline reagent	+
	Shinoda	+
Saponin	Foam	-
Triterpenoids and steroids	Salkowski	+
	Libermann-burchard	+
Tannin and phenolic compounds	Ferric chloride	+
	Lead acetate	+
	Dilute iodine solution	+

Note: +ve – positive, -ve – negative.

DISCUSSION: The DPPH assay is a very simple method for screening small anti-oxidant molecules, because the reaction can be observed visually and intensity can be analyzed using common spectrophotometric assay.

The stable radical DPPH has been used widely for the determination of primary anti-oxidant activity. The DPPH anti-oxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of anti-oxidants. Qualitative and quantitative phytochemical analysis showed that the plant is rich in flavonoids and phenols that are considered to be potent anti-oxidants, so this activity may be attributable to the presence of these chemicals in the extract. Literature survey has shown that Ganiyu Oboh (2006)[24] studied the antioxidant activity of ethanolic extract of its leaf by DPPH assay, in which the extract showed 51.2 % inhibition. Thus to the best of our knowledge and in accordance with the literature survey, this is the first report in which ethanolic extract of O.G. leaf at a concentration of 200µg/ml has shown a high % inhibition of 82.40% by DPPH assay.

CONCLUSION: The *in vitro* results should be confirmed in vivo so as to develop potent antioxidant from this plant, as this property of the extract will be advantageous in preventing oxidative stress and in preventing the various other diseases caused by it. Further the extract could be fractioned and fractions could be analyzed for extracting the particular active principle responsible for this activity.

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