Research Article

Evaluation of Antioxidant Potential in *Ocimum Americanum*

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ABSTRACT

Medicinal and Aromatic plants, a gift of nature produce a diverse array of primary and secondary metabolites known as phytochemicals that have antioxidant properties. Hence the present study is aimed at investigating the Antioxidant potential and phytochemical screening in *Ocimum americanum*. The powdered plant sample of *Ocimum americanum* was extracted with various buffers and the extracts were analysed for the enzymatic and nonenzymatic antioxidants. The *Ocimum americanum* was found to exhibit all the enzymatic antioxidants like catalase, peroxidase, superoxide dismutase, glutathione peroxidase, and polyphenol oxidase and nonenzymatic antioxidants like ascorbic acid, α-tocopherol, reduced glutathione and polyphenol. Phytochemical screening of aqueous and ethanolic extract of the leaves of *Ocimum americanum* was carried out. The present study reveals that the leaf part of *Ocimum americanum* appears to be a good source of antioxidants and phytochemicals.

Keywords: *Ocimum americanum*, antioxidants, peroxide.

INTRODUCTION

Today there is a renewed interest in traditional medicine and an increasing demand for more drugs from plants sources. This revival of interest in plant –derived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than the costly synthetic drugs. Natural plants are known to play an important role in pharmaceutical biology. Plants have been an important source of medicine for thousands of years. Even today, World Health Organization estimated that up to 80% of people still remain on traditional medicines. In fact, many of the current drugs either mimic naturally occurring molecules or have structures that are fully or in part derived from natural motifs[1].

Natural products and secondary metabolites formed by living systems, notably from plant origin, have shown great potential in treating human diseases such as cancer, coronary heart diseases, diabetes and infectious diseases[2].

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism underlying a number of human neurodegenerative disorders, diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders[3].

Free radicals are generated through normal metabolism of drugs, environmental chemicals and other xenobiotics as well as endogenous chemicals, especially stress hormones (adrenalin and noradrenalin)[3]. Reactive oxygen species (ROS) are a class of highly reactive molecules derived from the metabolism of oxygen. ROS, including superoxide radicals, hydroxyl radicals and hydrogen peroxide molecules are often generated as by-products of biological reactions or from exogenous factors. There is extensive evidence to involve ROS in the development of degenerative diseases. Evidence suggests that compounds especially from natural sources are capable of providing protection against free radicals. This has attracted a great deal of research interest in natural antioxidants. It is necessary to screen out medicinal plants for their antioxidant potential[4].

In recent years, there has been growing interest in finding natural antioxidants in plants because they inhibit oxidative damage and may consequently prevent aging and neurodegenerative diseases[5]. Many medicinal plants contain large amount of antioxidants, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The medicinal value of plant has assumed a more important dimension in the past few decades owing largely to the discovery that extracts from plants contain not only minerals and primary metabolites but also a diverse array of secondary metabolites with antioxidant potential[6].

Ocimum is one of the most important genus of the Lamiaceae family, due to the extensive use of many of its species as economically important medicinal and culinary plants. According to ethnobotanical information people from Northeastern Brazil have been using infusions of *Ocimum* species for ritualistic aromatic baths, and as a tea for treating gastro-intestinal problems and also for seasoning special foods[7]. Numerous studies reported various effects of *Ocimum* sp., including antiinflammatory, antioxidative, chemopreventive, blood-sugar lowering, nervous system stimulation and radiation protection have been reported[8].

MATERIALS AND METHODS

Collection of the plant material

The leaves of the plant sample were collected from in and around the Nilgiri district of Tamil Nadu. Fresh plant leaves rinsed with clean tap water to make it dust and debris free. Then the leaves were spread evenly and dried in a shady condition for 5 to 6 days, until they become crispy while still retaining the green coloration. The dried leaves were coarsely powdered and used for the experiment.

Determination of the antioxidant potential

The powdered plant material was extracted with various buffers and the extracts were analysed for the enzymatic and nonenzymatic antioxidants.

Enzymatic antioxidants

Estimation of catalase

Catalase activity was estimated by the method of Luck *et al*., (1974)[9]. The sample was homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay. Read against a control cuvette 3ml of H_2O_2 containing the enzyme solution as in the phosphate buffer (M/15). Pipetted into the experimental cuvette 3ml of H_2O_2 phosphate buffer. Mixed in 0.01-0.04ml sample with the glass or plastic rod flattened at one end. Noted the time it required for a decrease in absorbance from 0.45-0.4. This value was used for calculations. If 't' was more than 60 seconds, repeated the measurement with more concentrated solution of the sample. Calculated the concentration of H_2O_2 using the extinction coefficient 0.036µ mole/ml.

Estimation of peroxidase

Peroxidase activity was estimated by the method of Reddy *et al*., (1995)[10]. Macerated one gram of the sample with 5 ml (w/v) 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuged the homogenate at 3000 rpm for 15min. used the supernatant as the enzyme source. All procedure was carried out at 0-5°C. Pipetted out 3ml of 0.05 M pyrogallol solution and 0.5 to 1.0 ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400 nm. Added 0.5 ml of 1% hydrogen peroxide in the test cuvette. Recorded the change in the absorbance every 30 seconds upto 3 minutes.

Change in absorbance / min = X

Weight of the plant material taken = 1 g

Volume of the extract taken for the assay = 0.02 ml

Change in absorbance for 1.5 ml extract = (X / 0.02) x 1.5 – Y

(i.e) Peroxidase activity in 300 mg plant tissue = Y

Peroxidase activity / g of plant tissue = Y x (1000/300) Units

Estimation of superoxide dismutase

Superoxide dismutase activity was estimated by the method of Misra and Fridovich (1972)[11]. The incubation medium contained a 300µl of each reagent (50mM potassium phosphate buffer (pH 7.8), 450 µM Methionine, 53mM Riboflavin, 840µM Nitro Blue Tetrozolium (NBT), and 200µM potassium cyanide. To the test 300µl of sample was added. The final volume was made up to 3ml with water. The tubes were placed in an aluminum Foil lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophtomerically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of enzyme giving 50% inhibition of the reduction of NBT.

Estimation of glutathione peroxidase

Glutathione peroxidase activity was estimated by the method of Rotruct *et al*., (1973)[12]. To 2ml of Tris buffer, 0.2 ml of EDTA, 0.1ml of sodium azide and 0.5 ml of plant extract were added followed by 0.1 ml hydrogen peroxide were added to the mixture, mixed well and incubated at 37°C for 10 minutes along with the tube containing the entire reagent expect sample. After 10 min the reaction was arrested by the addition of 0.5ml of 10% TCA centrifuged and supernatant was assayed for glutathione by the method of Ellman.

The activities are expressed as µg GSH consumed / min / mg protein.

Estimation of polyphenol oxidase

The poplyphenol oxidase activity was estimated by the method of Esterbauer *et al*., (1997)[13]. Added 2.5ml of 0.2M phosphate buffer (pH 6.5), 0.3ml of catechol solution (0.01 M) into the cuvette and set the spectrophotometer at 495nm. Now add the enzyme extract (0.2ml) and started recording the change in absorbance for every 30 seconds up to 5 minutes

Enzyme units in the test = K *(Δ / min)

K for catechol oxidase = 0.272

K for laccase = 0.242

Non enzymatic antioxidants

Estimation of ascorbic acid (vitamin C)

Ascorbic acid was estimated by the method Roe and Keuther (1953)[14]. 1g of the sample was homogenized in 1% TCA up to 10ml. Centrifuged at 2000rpm for 10 minutes. To the supernatant, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. Centrifuged again and removed the charcoal residue. The volume of the clear supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay.

The assay volumes were made up 2.0ml with 1%TCA. 0.2 to 1.0ml of the working standard solution of 20-100 µg of

ascorbate respectively were pipetted out into test tubes. Added 0.5ml of DNPH to all the tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 hrs. The osazones formed were dissolved in 2.5ml of 85% sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank, DNPH and thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

Estimation of α-tocopherol (vitamin E)

α-Tocopherol was estimated by the method of Rosenburg (1992)[15]. Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5ml of extract, 1.5ml of standard, 1.5ml of water respectively. To test and blank added 1.5ml of ethanol and to the standard added 1.5ml of water. Added 1.5ml xylene to all the tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube. Added 1.0ml of 2, 2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixture into colorimeter cuvettes and read the extinction of test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula.

$$\text{Amount of tocopherols} = \frac{\text{Reading at 520nm- Reading at 460nm}}{\text{Reading of standard at 520nm x 0.29 x 15}}$$

Estimation of polyphenols

Polyphenol activity was estimated by the method of Malik and Singh (1980)[16]. 1g of sample was homogenized using 20ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The residue was reextracted with 10ml of 80% ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (50ml) and 2.0ml was taken for the experiment. A working standard of 0.5 – 2.5ml catechol solution corresponding to 50 - 250µg of catechol were pipetted out into a series of test tubes. The volume was made up to 2.5ml with water. To all the tubes added 0.5ml of diluted Folin – ciocalteau reagent. After 3 minutes, added 2.0ml of 20% Na_2CO_3 solution to each tube and mixed thoroughly.

The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650nm against a reagent blank. Constructed a standard graph by plotting the concentration of catechol on X-axis and absorbance on Y-axis. From the graph, the amount of polyphenols present in the sample was estimated and expressed as mg of polyphenols per g of the sample.

Estimation of reduced glutathione

Reduced glutathione content was estimated by the method of Moron *et al*., (1979)[17]. 1g of the sample was homogenized in 5%TCA to give a 20% homogenate. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0), 2ml of freshly prepared DTNB solution (0.6mM) in 0.2M phosphate buffer (pH 8.0) was added to the tubes and intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 minutes.

A standard curve of GSH was prepared using concentration ranging from 2 to 10 nmoles of GSH in 5%TCA.

Identification of Phytochemicals

Phytochemical screening of aqueous and ethanolic extract of the leaves of *Ocimum americanum* was carried out.

Alkaloids

50 mg of solvent free extract was stirred with one ml of dilute hydrochloric acid and filtered. The filtrate was tested for alkaloids[18].

Mayer's Test: To the filtrate, a drop of Mayer's reagent was added along the sides of the test tube. A white precipitate indicates the test as positive.

Flavonoids

Alkaline reagent test: Two ml of aqueous solution of the extract was treated with 1 ml of 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids[19].

Saponins

50 mg of the plant extract was ground with 3 ml of distilled water and diluted with the same, made upto 20ml. The suspension was shaken in a graduated cylinder. After 15 min, a two cm layer of foam indicated the presence of saponins[18].

Phenols

Ferric chloride test: 50mg of the sample was dissolved in 5ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds[20].

Carbohydrates

To 0.5ml of the extract of the plant sample, 1ml of water and 5-8 drops of Fehling's solution was added at hot and observed for brick red precipitate[21].

Protein

To 1 ml of the extract few drops of Barfoed's reagent was added to give blue color products[20].

Tannins

One ml of water and 1-2 drops of ferric chloride solution was added to 1 ml of extract of the plant sample. Blue colour was observed for gallic tannins and green black for catecholic tannins[18].

Steroids

Libermann-Burchard reaction: 4mg of the plant extract was treated with 0.5 ml of acetic anhydride and 0.5ml of chloroform. Then concentrated sulphuric acid was added slowly and green bluish colour for steroids was observed[19].

Volatile Oils

Shake 2ml of the extract solution with 0.1ml dilute sodium hydroxide and a small quantity of dilute HCl. Formation of white precipitate indicates the presence of volatile oils.

RESULTS AND DISCUSSION

Identification of Phytochemicals

From the Table 1 it is evident that steroids and alkaloids are positive for ethanolic extract (positive for Dragondroff's). Both the extracts contain carbohydrates (positive for starch and cellulose). Protein, phenol, quinone, flavonoids, tannins and volatile oils are present in both aqueous and ethanolic extracts. The phenolic compounds have potentially beneficial effects on human health by reducing the occurrence of coronary heart disease, age-related eyes diseases, and artherogenic processes. These compounds also have antioxidant and antifree-radical properties that allow them to quench free radicals in the body. Moreover, it was reported that antioxidants with ROS scavenging ability have great relevance in the prevention of oxidative stress which is responsible for majority of the diseases[22].

Antioxidant Potential

Enzymatic antioxidants

From the Table 2 it is evident that the leaf sample exhibits all the above enzyme activities to different levels. The activity of catalase was found to be 349.3±173.52U/g while that of peroxidase was 0.197±0.037 U/g. The other antioxidant enzymes such as Superoxide dismutase, Glutathione peroxidase and Polyphenol oxidase have recorded activities 29.95±4.692 U/g, 1.128±0.289 U/g and 0.107±0.002 U/g respectively.

The activities of the antioxidant enzymes (catalase and superoxide dismutase) were found to be significantly higher in the liver of Basil (*Ocimum basilicum* and *Ocimum tenuiflorum*) fed rats compared to the control rats indicating that these are good sources of these enzymic antioxidants[23]. The activities of CAT and SOD were restored almost to the normal condition in alcohol induced experimental rats administered with methanol extract of *Ocimum gratissimum* and *Ocimum canum*. These findings again support the participation of these two plants in maintaining the normal antioxidant system[24].

Non enzymatic antioxidants

Table 3 represents the various non enzymatic antioxidants such as ascorbic acid, reduced glutathione, α-tocopherol and polyphenol. In the present study the assessed ascorbic acid level is 0.946 (mg/g) and reduced glutathione is 1.17 (nmole/g). The levels of α-tocopherol and polyphenol are 0.0104 and 0.1079 mg/g respectively. *Mentha pulegium* and *Thymus pulegioides* plants belonging to the family Lamiaceae showed the highest levels of tocopherols, particularly α-tocopherol and ascorbic acid[25]. Elevated levels of reduced GSH in liver, lung and stomach tissues of mice supplemented with *Ocimum sanctum* leaf extract was reported by Prashar R. and Kumar A. (1995)[26].

Table 1: Identification of phytochemicals in the leaf extract of *Ocimum americanum*

Phytochemicals	*Ocimum americanum* (Leaf extract)	
	Aqueous	Ethanol
Carbohydrates		
a) Starch	+	+
b) Cellulose	+	+
Protein	+	+
Steroid	-	+
Phenol	+	+
Saponins	-	-
Quinone	+	+
Alkaloids		
a) Dragondroff's test	-	+
b) Wagner's test	-	-
Flavonoids	+	+
Tannins	+	+
Volatile oils	+	+

+ indicates presence: - indicates absence

Table 2: Activities of enzymatic antioxidants in the leaves of *Ocimum americanum*

Enzymatic antioxidants (U / g of sample)				
Catalase Units */g	Peroxidase Units **/g	SOD Units***/g	GPx Units +/g	PPO Units ++/g
349.3±173.52	0.197±0.037	29.95±4.6925	1.128±0.289	0.107±0.002

Values represent Mean±S.D of three replicates; *Unit – Amount of enzyme required to decrease the absorbance by 0.05 units at 240nm
**Unit-Change in absorbance / min / g of sample; ***Unit-A mount of enzyme that gives 50% inhibition of the extent of NBT reduction
+ Unit- mill moles of CDNB-GSH conjugate / min / g; + + Unit- nmoles of GSH oxidized / min / g

Table 3: Activities of non-enzymic antioxidants in the leaves of *Ocimum americanum*

Non-enzymatic antioxidants			
Ascorbic Acid (mg/g)	**Reduced glutathione (nmoles)**	**α-tocopherol (µg/g)**	**Polyphenol (mg/g)**
0.946±0.25	1.17±0.33	0.0104±0.001	0.108±0.03

Values are mean ± S.D of three replicates

In the present study, the qualitative analysis of phytochemicals revealed the presence of carbohydrates, protein, steroids, phenol, quinone, alkaloids, flavonoids, tannins and volatile oils in the leaf extract of *Ocimum americanum*. The leaf sample of *Ocimum americanum* was found to exhibit activities for enzymatic antioxidants such as catalase, peroxidase, superoxide dismutase, glutathione peroxidase and polyphenol oxidase and non enzymatic antioxidants such as ascorbic acid, reduced glutathione, α tocopherol and polyphenol. In conclusion, the present findings reveal that, the leaf part of *Ocimum americanum* appears to be a good source of antioxidant.

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