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Archives • 2013 • vol.3 • 37 - 44

In Vitro Antioxidant Activity and Estimation of Total Phenolic Content in Ethyl Acetate Extract of *Ocimum Gratissimum*

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Abstract

Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human against degenerative diseases. Current research is now directed towards natural antioxidants originated from plants due to safe therapeutics. *Ocimum gratissimum* is used in traditional medicine for a wide range of various ailments. To understand the mechanism of pharmacological actions and investigate the antioxidant activity of the plant. The antioxidant properties of the *Ocimum gratissimum* ethyl acetate extract were tested using standard *in vitro* models and total phenolic content. The antioxidant activity of the extract increased in a dose dependent manner as well as the reducing power indicating some compounds in *Ocimum gratissimum* is both electron donors and could react with free radicals to convert them into more stable products and to terminate chain reactions. The extract had IC_{50} values for chelating effect of to be 5.51 mg/ml. In DPPH radical scavenging assay, the IC_{50} value was 1.58 mg/ml. The extract was found to inhibit the nitric oxide radicals generated from sodium nitroprusside, the IC_{50} values for nitric oxide scavenging ability was 13.17 mg/ml. However, it was also found to inhibit the hydroxyl radical generated by the deoxyribose method, the IC_{50} values was 6.48mg/ml. The total phenolic contents of *Ocimum gratissimum* determined in this study were 10.34±0.47 mg GAE/g.This study suggests that the extract of *Ocimum gratissimum* have potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage.

KEYWORDS: OCIMUM GRATISSIMUM, ANTIOXIDANTS, PHENOLIC CONTENT, IN VITRO

Introduction

Free radicals are constantly generated in vivo for physiological purposes [1]. They can be overproduced in pathological conditions, causing oxidative stress [2]. A large number of civilization associated diseases such as autoimmune diseases, inflammation, cardiovascular-neurological diseases, cancer and aging are attributed to oxidative stress [3, 4]. An adequate intake of natural antioxidants could protect macromolecules against oxidative damage in cells [5]. The term antioxidant refers to free radical scavengers, inhibitors of lipid peroxidation and chelating agent [6].

Phenolic compounds possess a wide spectrum of biological effects including antioxidant and free radical scavenging [7, 8]. In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers [9, 10].

The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities [11]. Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic [12]. They were also suggested to be a potential iron chelator [13, 14]. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties. *Ocimum gratissimum* Linn (Labiatae) is grown for the essential oils in its leaves and stems.

Eugenol, thymol, citral, geraniol and linalool have been extracted from the oil [15]. Essential oils from the plant have been reported to possess an interesting spectrum of antifungal properties [16]. The antinociceptive property of the essential oil of the plant has been reported [17]. Therefore, the objective of the study is to evaluate the *in vitro* antioxidant potential of extracts from the leaves of *O. gratissimum* and to explore the basis for its traditional use.

Methods

Collection of plant

Fresh leaves of *O. gratissimum* were bought in the market at Ado, Nigeria. The plant was identified and authenticated by a plant scientist in the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria and a voucher specimen was deposited accordingly at the herbarium of the Department of Plant Science.

Preparation of extract

Ethyl Acetate preparation

The ethyl acetate extract of the powered *Ocimum gratissimum* leaves were prepared using the method of Hong *et al*. [18]. The percentage yield of extraction calculated as follows was 2.08 %.

$$\% \text{ yield} = \frac{\text{Weight of the dry extract}}{\text{Weight of powdered leaves}} \times 100\%$$

Determination of Total phenolic content

The extractable phenol content were determined on the extracts using the method reported by [19]. Appropriate dilutions of the extracts were mixed with 2.5 mL of 10% Folin–Ciocalteau's reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixtures were incubated for 40 min at 45 °C and the absorbance were measured at 765nm in the spectrophotometer. The total phenol content were subsequently calculated using gallic acid as standard. The concentrations of total phenols were expressed as mg/gm of dry extract.

Determination of Reductive ability

The reducing property of the *Ocimum gratissimum* extracts were determined by assessing the ability of the extract to reduce a $FeCl_3$ solution as described by [20]. A 2.5 ml aliquot were mixed with 2.5 ml, 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml, 1% potassium ferricyanide. The mixtures were incubated at 50 °C for 20 min, and then 2.5 ml, 10% TCA were added. These were then centrifuged at 650 g for 10 min. A 5 ml of the supernatant were mixed with an equal volume of water and 1 ml, 0.1% ferric chloride.

Determination of DPPH radical scavenging activity

The free radical scavenging activity of the hydroalcoholic extract of *Ocimum gratissimum* was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Blois; a method based on the reduction of a methanolic solution of the coloured DPPH radical [21, 22]. After 30 minutes of incubation, absorbance was measured at 517 nm. The percentage reduction in absorbance was calculated from the initial and final absorbance of each solution [23, 24]. The percentage inhibition was calculated by comparing the absorbance values of control and samples. Percentage scavenging of DPPH radical was calculated using the formula,

$$pS = \frac{Ac - At}{Ac} \times 100$$

Ac = Absorbance of Control
At = Absorbance of Test
pS = % Scavenging of DPPH

Determination of Iron Chelation Ability

The Fe^{2+} chelating ability of both extracts were determined using a modified method of [25] with a slight modification by [26]. Freshly prepared 500 µmol L^{-1} $FeSO_4$ (150 µL) were added to a reaction mixture containing 168 µL of 0.1 mol L^{-1} Tris–HCl (pH 7.4), 218 µL saline and the extracts (0–25 µL). The reaction mixtures were incubated for 5 min, before the addition of 13 µL of 0.25% 1, 10-phenanthroline (w/v). The absorbances were subsequently measured at 510 nm in a spectrophotometer. The Fe^{2+} chelating ability were calculated with respect to the control.

$$pC = \frac{Ac - At}{Ac} \times 100$$

pC = Percentage Fe^{2+} chelating ability (%)
Ac = $Abs_{control}$
At = $Abs_{test\ sample}$

where $Abs_{control}$ = absorbance of the control (reacting mixture without the test sample) and, $Abs_{test\ sample}$ = absorbance of reacting mixture with the test sample.

Determination of Hydroxyl radical scavenging effect

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compounds for hydroxyl radical generated by Fe^{3+}-Ascorbate-EDTA H_2O_2 system (Fenton reaction) according to the method of [27]. The hydroxyl radicals attack deoxyribose that eventually results in TBARS formation. The free radical damage imposed on the substrate, deoxyribose was measured as TBARS by the method of [28] 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) were added to the test tubes and the incubation was continued at 100 °C for further 20 min. After cooling, absorbance was measured at 532 nm against control containing deoxyribose and buffer.

Nitric oxide radical scavenging effect

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by the Griess reaction. [29, 30] Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide [31]. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline

(PBS) and the extract in different concentrations (1, 2, 3, 4 and 5 mg) were incubated at 25 °C for 150 min. At every 30 min interval, 0.5 mL of the incubated sample was removed and 0.5 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO4) was added. The absorbance of the chromophore formed was measured at 546 nm.

Statistical analysis

Statistical analysis of difference between groups was evaluated by one-way ANOVA followed by student t test. The values $P < 0.05$ were regarded as significant.

Results

The total phenolic content of ethyl acetate extract of *Ocimum gratissimum* is 10.34±0.47 mg GAE/g.

The ethyl acetate extracts of *Ocimum gratissimum* show a high reducing power as seen in (figure 1). IC_{50} value for reducing power by the ethyl acetate extract of *O. gratissimum* was 1.55mg/ml. This result shows that the ethyl acetate extract of *O. gratissimum* has a high reducing power.

The scavenging effect of extracts in the range 1–5 mg/ml on the DPPH radical increased with an increasing concentration of OG extracts (figure 2). IC_{50} value for DPPH scavenging by the ethyl acetate extracts of *O. gratissimum* were 1.58mg/ml. In the present investigation *Ocimum gratissimum* at different doses demonstrated significant DPPH scavenging activity indicating their abilities to act as radical scavengers.

Ocimum gratissimum ethyl acetate extract had higher chelating effect. IC_{50} values for chelating effect of ethyl acetate extracts of *O. gratissimum* were 5.51mg/ml. This shows that ethyl acetate extract of *O. gratissimum* has a higher chelating ability.

Any hydroxyl radical scavenger added to the reaction would compete with deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation. IC_{50} values for hydroxyl radical effect of ethyl acetate extracts of *O. gratissimum* were 6.48mg/ml.

The extract competes with oxygen to react with nitric oxide and thus inhibits generation of the anions. Figure 5 illustrates the percentage inhibition of nitric oxide generation by the different form of extract of *Ocimum gratissimum*. The extract of *Ocimum gratissimum* showed significant free radical scavenging activity on nitric oxide (NO)-induced release of free radicals. From Figure 5, the concentration of the extract increased, as the absorbance decreased. IC_{50} values for nitric oxide scavenging ability of ethyl acetate extracts of *O. gratissimum* were 13.17mg/ml.

Discussion

Total phenolic compounds are reported as pyrocatechol equivalents. It has been reported that green leafy vegetables, soft fruits and medicinal plants exhibited higher levels of flavonoids [32]. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities [33]. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700nm [34]. DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts [35]. DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow coloured product, diphenyl picryl hydrazine, with the addition of the fractions in a concentration-dependent manner. It is usually used as a substrate to evaluate the antioxidative activity of antioxidants [36]. DPPH scavenging activity has been used by various researchers as a quick and reliable parameter to assess the *in vitro* antioxidant

activity of crude plant extracts [37, 38]. In the present investigation *Ocimum gratissimum* at different concentration demonstrated significant DPPH scavenging activity indicating their abilities to act as radical scavengers. Iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation [39]. Metal ions play an important role in the acceleration of oxidation of important biological molecules, for instance they may catalyze the formation of first few radicals that can lead to propagation of the radical chain reaction in lipid peroxidation [40]. This indicates that the chelation property of the extracts on the Fe^{2+} ions may be able to afford protection against oxidative damage.

It was reported that chelating agents, which form d-bond with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion [40], Chelating agents inhibit the radical mediated oxidative chain reactions in biological or food systems, and consequently improve human health, and food quality, stability and safety. Citric acid and its salts phosphates and salts of EDTA are among the most commonly used chelators. In addition, plant phenolic compounds have also been found to be good metal ion chelators [41].

Hydroxyl radicals are the major active species causing lipid oxidation and enormous biological damage [42, 43]. The deoxyribose method is a simple assay to determine the rate constants for reactions of hydroxyl radicals [44]. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose in to fragments that on heating with TBA at low PH form a pink chromogen [45, 46].

In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent [47].

Conclusion

On the basis of the results obtained in the present study, it is concluded that ethyl acetate extract of *Ocimum gratissimum* leaf exhibits high antioxidant and free radical scavenging activities. It also chelates iron and has reducing power. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use.

Acknowledgments

The Authors wish to acknowledge Mr. Omotayo of the Department of Plant Science, Ekiti State University, in helping to identify the plant and providing the voucher number as well as Afe Babalola University Biochemistry Laboratory where the research was conducted.

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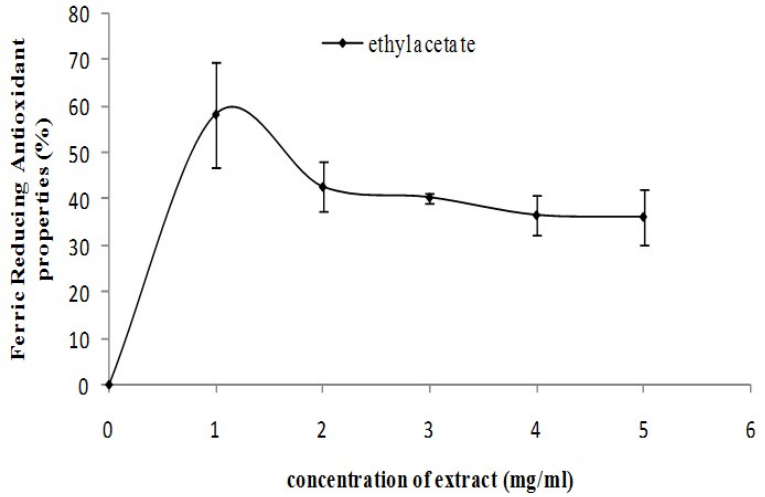

Figure 1: Ferric reducing antioxidant properties of ethyl acetate extract of *Ocimum gratissimum*.

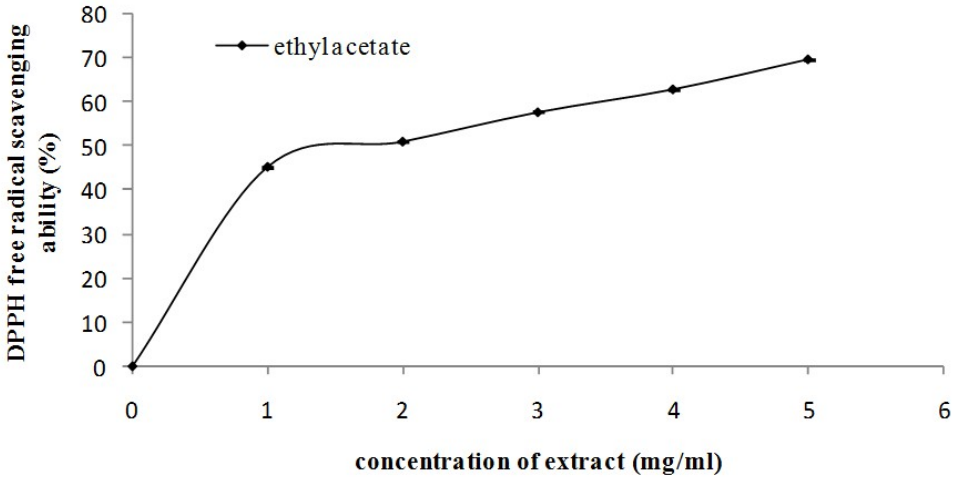

Figure 2: DPPH free radical scavenging ability of ethyl acetate extract of *Ocimum gratissimum*.

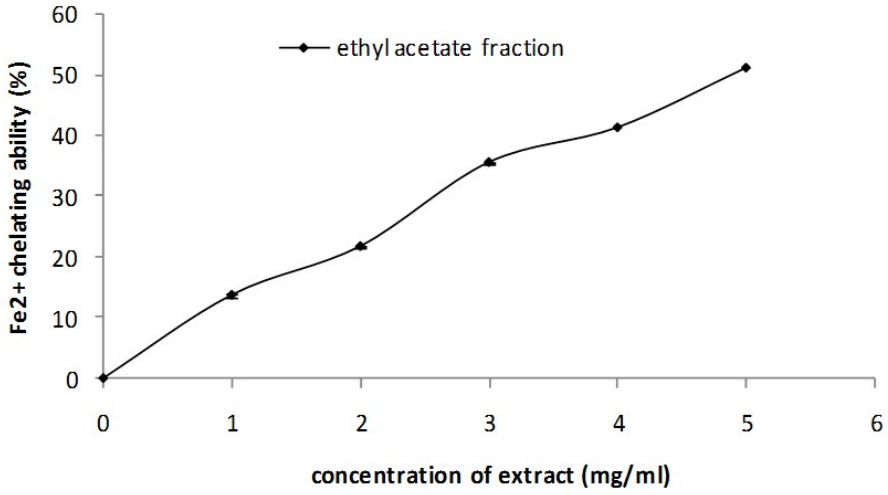

Figure 3: Iron chelating ability of ethyl acetate extract of *Ocimum gratissimum*.

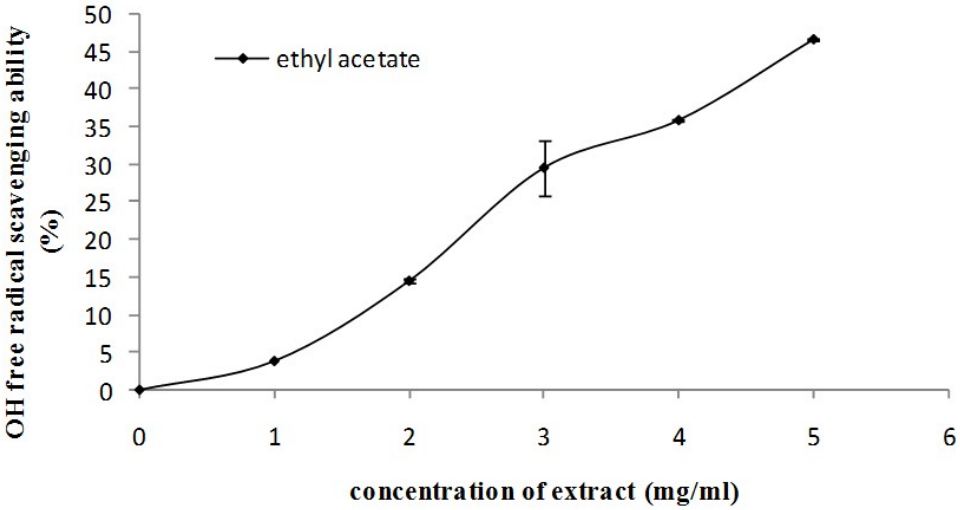

Figure 4: Hydroxyl free radical scavenging ability of ethyl acetate extract of *Ocimum gratissimum*.

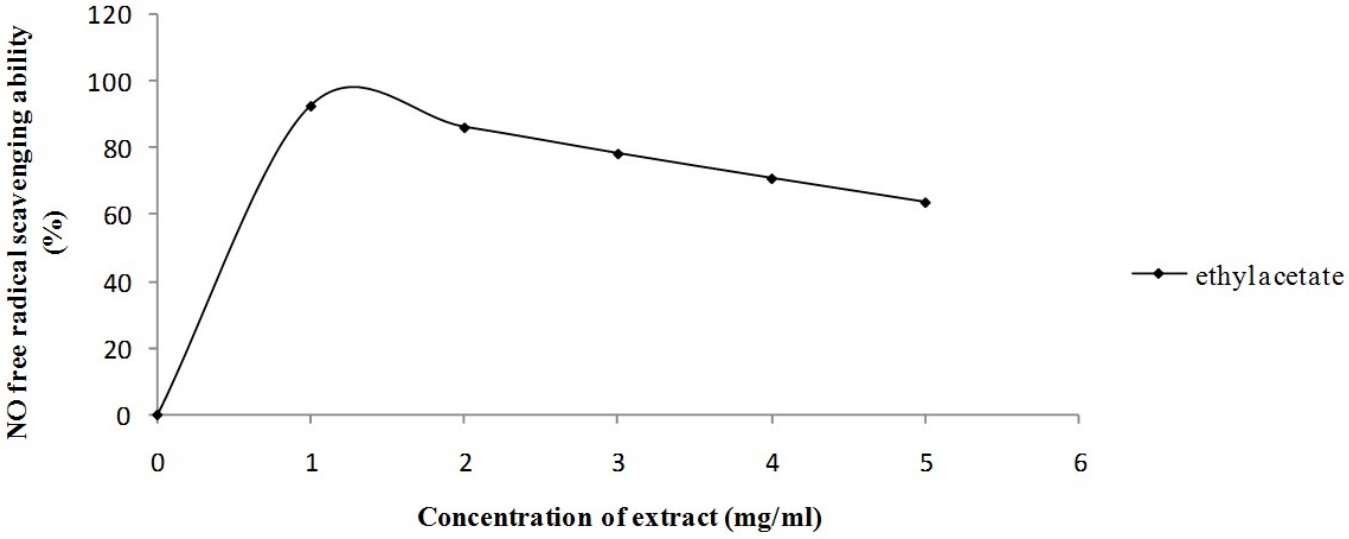

Figure 5: Nitric oxide free radical scavenging ability of ethyl acetate extract of *Ocimum gratissimum*.