ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Determination of polyphenolic content and antioxidant activities of essential oil of *Ocimum sanctum*, L

Sathaye S* and Redkar RG

Pharmacology Research Laboratory-II, Department of Pharmaceutical Sciences & Technology (Now UGC-CAS)
Institute of Chemical Technology, Matunga, Mumbai-400 019, India

ABSTRACT

The essential oil hydro distilled from the fresh leaves of *Ocimum sanctum*, Linn was studied for its individual chemical components by GC-MS. The yield of essential oil was found to be 0.31 ml /100 g of fresh leaves with methyl eugenol ether as the chief constituent. It was investigated for its antioxidant and free radical scavenging activities including [1,1-diphenyl-2-picryl hydrazyl radical] DPPH, nitric oxide scavenging activity, iron chelatory potential and (2, 2'-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid]) **ABTS** assay. The results suggest that the polyphenolic content in the essential oil was found to be 257.1 mg equivalent antioxidant capacity (EAC)/g of oil. The DPPH inhibitory and nitric oxide (NO) radical scavenging activities measured in terms of their IC_{50} of 0.149 mg/ml and 0.175 mg/ml respectively indicated its antioxidant activity. The total antioxidant activity (TAA) of the essential oil determined by the ABTS assay was 123 EAC/µg of the sample. The iron chelatory potential revealed by percentage ferrous-ferrozine formation inhibition was at IC_{50} of 3.21 mg/ml; exhibited at much higher concentration. There was a direct significant correlation between concentrations of essential oil and its antioxidant activities as percentage inhibition of free radicals and metal chelation.

Keywords: *Ocimum sanctum*, essential oil, polyphenolics, free radicals, antioxidants, iron chelatory activity

***Corresponding author**
Email: sadhanasathaye@gmail.com

ISSN: 0975-8585

INTRODUCTION

Overproduction of reactive oxygen and nitrogen species (ROS and RNS) in a biological system leads to oxidative and nitrosative stresses and decreased total antioxidant capacity, which is responsible for high mortality from several diseases [1]. Oxidative stress results from an oxidant/antioxidant imbalance, an excess of oxidants and/or a depletion of antioxidants. Free radicals have also been implicated in degenerative diseases including cardiovascular ailments, neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and multistage process of carcinogenesis [2, 3]

In the nitrosative stress, nitric oxide (NO) is a gaseous free radical which has important roles in physiological and pathological conditions. Marcoci et al have reported that scavengers of NO compete with oxygen, leading to a reduction in the production of NO. The reaction between nitric oxide (NO) and superoxide radical (O2•−) produce the oxidant peroxynitrite (ONOO−), the latter which is more hazardous [4]

The harmful action of the free radicals can, however be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism. [5, 6] Insufficient entry of antioxidants in human organism can lead to the biological structure damages: DNA, lipids and proteins [7]. Pro-oxidant/antioxidant balance is also crucial in neurodegenerative processes, including cell death, motor neuron disease, and axonal injury and antioxidants directly or indirectly protect cells from adverse effects of xenobiotics, drugs, carcinogens and toxic chemical reactions [8].

Most antioxidants in plants are phenolics which act as chain-breaking antioxidants. Phenols sometimes have additional mechanisms of antioxidant action, e.g. by chelating transition metal ions [9]. Phenolics are a group of non-essential dietary components that have been associated with inhibition of atherosclerosis, and cancer, by chelating metals, inhibiting lipooxygenase and scavenging of free radicals [10]. These effects have been attributed to antioxidant components such as plant phenolics, flavonoids, and phenylpropanoids among others. Recent studies have shown that polyphenols possess potential effects of neuroprotection [11].

Increased levels of iron Fe (II) enhance the conversion of H_2O_2 to hydroxyl radical (.OH) via the Fenton reaction and favor a great turnover in the Haber-Weiss cycle, which leads to amplification of ROS [12]. Free radical scavengers and iron chelators would become increasingly important [13] to prevent and control the excessive production of ROS.

Ocimum sanctum, Linn (English: Holy Basil, Sanskrit: tulsi; Family: Labiatae) is a well-known traditional plant used in Ayurvedic system of medicine [14].Amongst the indigenous herbals of India, several therapeutic properties have been attributed to O. sanctum-a Medhya rasayanas.

The essential oils from Ocimum contain many terpenes (linalool, citral, 1, 8-cineole) and phenylpropanoids (e.g. methyl chavicol, eugenol) produced in specialized glandular trichomes [15].Presence of eugenol attributes to its anti-oxidative property and is also thought to be responsible for inhibition of lipid per oxidation [16]**.** This property helps in maintaining good health and in preventing the chances occurrence of heart diseases as well as most of the other biochemical diseases because oxidative stress is the hallmark of such diseases [17].

In addition, available literature data indicates that there is a great deal of diversity in the composition of essential oil of O. sanctum cultivated in different localities [18]. The present study was therefore aimed at determination of yield of volatile oil and its components, evaluation of the total phenolic content and antioxidant activity of the essential oil hydrodistilled from fresh leaves of O. sanctum, L that comes from the area of Vasai area in Thane district, Maharashtra to study the influence of geographical variability.

MATERIALS AND METHODS

Chemicals

All chemicals used in the assays were of analytical grade. Gallic acid, Folin Ciocalteau reagent, sodium carbonate, DPPH, ascorbic acid, sodium nitroprusside, sulfanilic acid reagent, naphthylethylene diamine dihydrochloride, 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid (ABTS), potassium persulphate, vitamin E acetate, ferrous chloride, ferrozine and disodium EDTA were obtained either from S.D Fine chemicals or Sigma- Aldrich.

Plant Material

The aerial parts of the herb *Ocimum sanctum*, L were procured from Vasai Market, Thane district, Maharashtra in the month of April (summer season) and authenticated at Khalsa College, University of Mumbai.

EXPERIMENTAL

Isolation of volatile oil

The fresh leaves were washed with water, hydro distilled using Clevenger apparatus and the essential oil was collected and stored at 4 C until being analyzed for its chemical components using GC-MS. The sample was diluted with hexane and subjected to GC-MS.

GC-MS Analysis

GC-MS was carried out with a Hewlett- Packard 6890/ Hewlett-Packard 5973 instrument. GC conditions were equipped on fused-silica capillary column 20 m × 0.25 mm i.d., 0.25 µm film thickness). Helium (at 0.5 ml/min) was used as a carrier gas. The injector was kept

at 240 °C and the transfer line at 280 °C. The column was maintained at 50 °C for 2 min and then programmed to 260 °C at 5 °C / min and held for 10 min at 260 °C. The MS was operated in the EI mode at 70 eV.

Compounds identification

Compounds were identified by comparing the gas chromatographic indices of the peaks i.e. the retention indices with literature values, [19] computer matching using the database and comparison with the fragmentation patterns of the mass spectra with those reported in the literature, NIST05 GC-MS libraries.

Determination of total polyphenolic content

It was carried out by Folin-Ciocalteau Method [20]. As linearity and absorbance for the Beer's Law was observed in the range of 50-700 µg/ml of gallic acid solution in water, different concentrations of 20, 60, 100,200,400,500 and 700 µg//ml were taken. They were suitably diluted with distilled water to 100 µl followed by 5.8 ml of additional amount of water and 500 µl of Folin's reagent. Then 1500 µl of freshly prepared sodium carbonate (aqueous) solution, mixed thoroughly and then incubated for 30-45 min in dark at 37 C. A gallic acid standard curve was obtained. Similarly diluted volatile oil was taken and treated in the same way. The blank readings were taken without gallic acid solution or test solution. The absorbance was measured at 760 nm using UV-visible spectrophotometer.

Phenolic content was expressed as mg standard equivalent/g of test.

Polyphenolic Content = Gallic acid equivalent (µg/ml) X volume of the sample X dilution factor X 10^{-6}(g) / wt of the sample.

Antioxidant activity by DPPH assay [1,1-diphenyl-2-picryl hydrazyl radical]

DPPH Assay is the widely used method for screening antioxidant potential of the sample as described by [21]. 0.5 ml of diluted essential oil at different concentrations was added to 0.5 ml of methanolic solution of DPPH (200 µM). Equal amount of methanol was added to the control. The samples were incubated at room temperature for 20 minutes and absorbance was recorded at 517 nm against blank.

% DPPH inhibition was calculated using the following formula:-

$$\% \text{ DPPH inhibition} = OD \text{ (Control)} - OD \text{ (sample)} \times 100 / O.D \text{ (Control)}$$

where OD is the Optical Density. Inhibitory concentration (IC_{50}) is used as a measure of comparison of antioxidant activity. It is calculated from the plot of % DPPH inhibition v/s concentration.

Determination of nitric oxide (NO) radical scavenging activity

NO scavenging activity was determined according to the method of [22]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO, which can be determined by the use of the Greiss Illosvoy reaction.

2 ml of 10 mM sodium nitroprusside in 0.5 ml of PBS (phosphate buffer saline pH 7.4) was mixed with 0.5 ml of test at various concentrations and the mixture incubated at 25^{o} C for 150 min. 0.5 ml was taken out from the incubated solution and added into 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated at R.T for 5 min. Finally, 1 ml of naphthylethylene diamine dihydrochloride (0.1% w/v) was mixed and incubated at RT for 30 min. The absorbance at 540nm was measured with a spectrophotometer.

The free radical scavenging activity was calculated as:

$$\% \text{ inhibition} = Ao\text{-}A_1 / Ao \text{ X } 100$$

where Ao = abs of the blank without test sample (control); A_1 = absorbance in presence of the test sample.

ABTS (2,2'-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid]) assay

The ABTS assay [23] was employed to measure the total antioxidant activity (TAA) of the sample. ABTS was dissolved in deionised water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (16 h) in the dark before usage. The resultant intensely-colored ABTS radical cation was diluted with 0.01 M PBS (phosphate buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The test was then diluted 100 X with the ABTS solution to a total volume of 1 ml. i.e. 10 µl sample + 990 µl of ABTS reagent.

Absorbance was measured spectrophotometrically at time intervals of 1, 5, 10, and 15 min after addition for a range of concentrations of the test. The assay was performed in triplicates. Controls without ABTS were used to allow for any absorbance of the test themselves, and 990 µl of PBS was added to these control samples instead.

The assay was first carried out on the water-soluble α-tocopherol (vitamin E) analogue, (Vitamin E acetate) concentration range of 5-200 µM which served as a standard. The results of the assay are expressed relative to the standard in terms of EAC (Standard equivalent antioxidant capacity).

ABTS value was calculated as using linear regression equation in the standard curve. The results of the ABTS assay are expressed in comparison to the standard i.e vitamin E.

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Iron chelating activity of essential oil of *Ocimum sanctum*, L

The chelating activity of the essential oil for ferrous ions was measured according to the method of Dinis et al [24] with slight modification.

To 1 ml of the diluted essential oil, 3.7 ml of methanol and 100 µl of ferrous chloride $FeCl_2$ (2 mM) was added. After 1 min, 200 µl of ferrozine was added and stirred on the vortex mixer. Ferrozine reacted with the divalent iron to form stable violet colored complex species that were very soluble in water. After 10 min, at RT, the absorbance of the Fe^{2+}- ferrozine complex was measured at 562 nm. Similarly absorbances of positive control (standard, disodium EDTA) was estimated.

The chelating activity of the extract for Fe^{2+} was calculated as:

Chelating activity % = Ao-As/ Ao X 100.

Where Ao = absorbance of control (blank, without test)
And As = absorbance's of test sample/ standard.

Statistical analysis

All the experiments were done in triplicate and the data subjected to statistical analysis using standard deviation of the mean. All data were represented as mean± SD

RESULTS AND DISCUSSIONS

Yield and components of essential oil by GC-MS

The essential oil obtained from hydrodistillation on fresh leaves of O. sanctum, L was in the yield of 0.31% v/w with characteristic odor. The crude essential oil of Tulsi is pale yellow oil and gave a deep bluish green coloration with alcoholic ferric chloride and is strongly phenolic in nature.

The essential oil was identified for its chemical components using GC-MS technique as shown by the GC-MS chromatogram in Fig.1.

Asha and coworkers have reported that the oil of O. sanctum possessed eugenol (53.10 %) as the main compound whereas Kothari and others (2004) [25] have reported methyl eugenol as the major constituent. Data from Thai species have revealed that O. sanctum, L that contained higher content of β-caryophyllene, linalool and higher yield of eugenol [26].

The yield of the oil (0.3-4.1%), which is highest from the leaves, and the chemical composition of the oil depend on geographical region and vary in vegetation period [27]. The oil

ISSN: 0975-8585

produced from plants grown in Cuba [28], India [29] and Germany [30] contained eugenol as a main constituent (34%, 53%, 24%, respectively), frequently together with a significant amount of β-caryophyllene or α- and β-bisabolenes. Methyl eugenol was the main constituent of some oils of O. sanctum from India (25%) [31] and Thailand (38-52%) [32], whereas the oil from plants grown in Australia was found to contain mainly methyl chavicol (87%) [33].

Table. 1: Chemical composition of essential oil obtained from the fresh leaves of O. sanctum, L

Peak	R.Time	I.Time	F.Time	Area	Area%	Components
1	2.386	2.337	2.475	568246	5.79	Eugenol
2	2.871	2.813	3.017	315541	3.22	isoeugenol methyl Ether
3	13.592	13.546	13.633	40872	0.42	Unidentified
4	14.924	14.817	15.029	1265233	12.90	caryophyllene
5	15.796	15.750	15.858	76256	0.78	humulene
6	16.094	16.050	16.133	35461	0.36	Unidentified
7	16.271	16.217	16.358	405788	4.14	borneol
8	16.863	16.746	16.933	121059	1.23	germacrene
9	19.423	19.346	19.696	6983174	71.17	methyl eugenol

R.time is the retention time, I.Time is the initial time and F.time is the final time

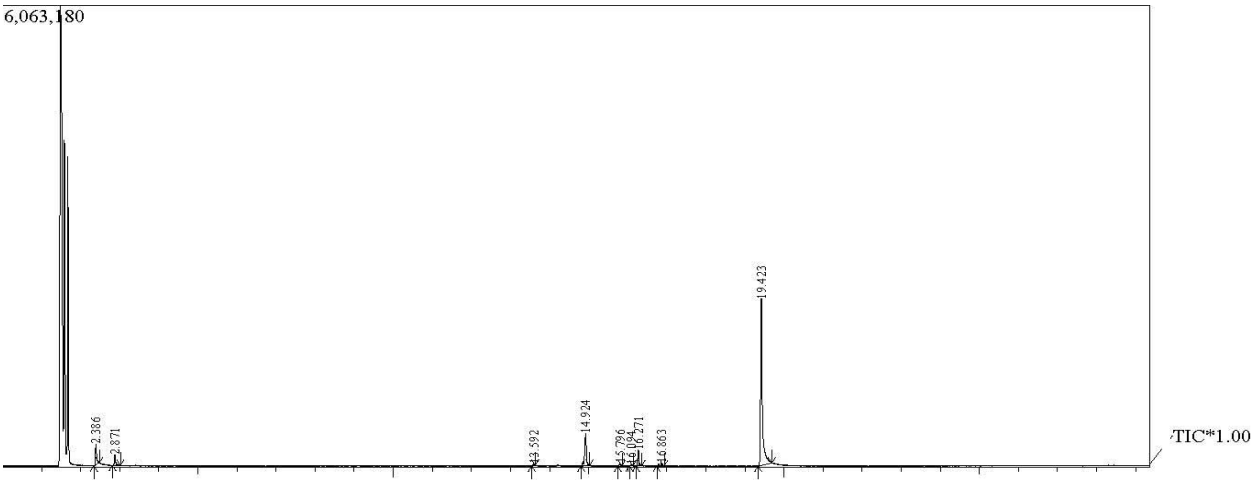

Fig.1. Chromatogram essential oil of *Ocimum sanctum*, L D:\gcmsdata\data\Others\Pharma\E. oil.qgd

The results as shown in Table.1 and Fig.1 revealed the presence of terpenoids and phenylpropanoids with methyl eugenol as a major constituent (71.17%) together with eugenol, iso eugenol methyl ether, caryophyllene, borneol, germacrene and humulene with other unidentified components. The results clearly imply that the climate and geographic conditions in different areas affects the essential oil production and composition.

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Determination of polyphenolics

Phenolics are the secondary metabolites that act as potential natural antioxidant (proton donors) for their ability to act as both efficient radical scavengers and metal chelator [34]. The total polyphenolics present in the essential oil was found to be 257.1 mg EAC/g of oil that was estimated in terms of gallic acid equivalents (GAE) correlated well with its antioxidant activity as in Fig.2.

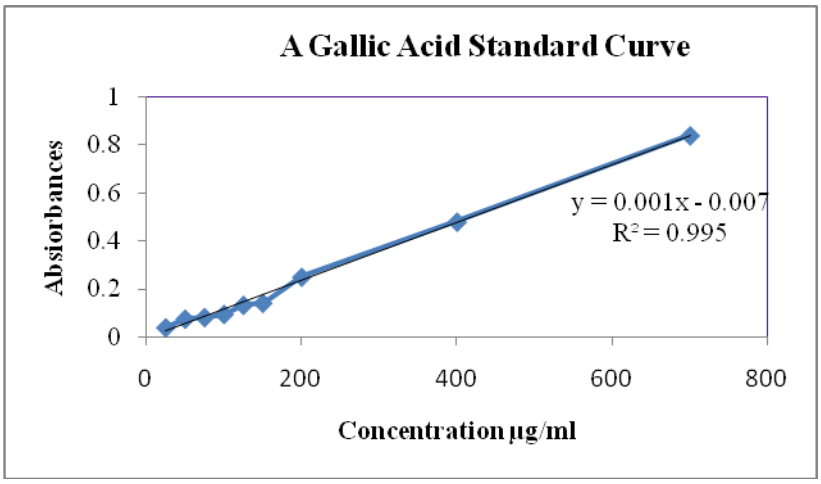

Fig.2. Gallic acid standard curve for determination of polyphenolic contents in gallic acid equivalents antioxidant capacity (EAC)

DPPH assay

The free radical scavenging capacity of essential oil was tested by its ability to bleach the stable DPPH radical [35]. He described a method involving use of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), where antioxidants are allowed to react with the stable radical in a methanol solution.

DPPH, a purple-colored stable free radical, was reduced into the yellow-colored diphenylpicryl hydrazine which is measured spectrophotometrically at 517 nm. It is the measure of the DPPH scavenging through reduction (protonation) of the DPPH radical to a more stable diamagnetic molecule of DPPHH stoichiometrically by an antioxidant compound.

The essential oil showed excellent antiradical activity as in Fig.3 by inhibiting DPPH radical with an IC_{50} value of 0.149 mg/ml in comparison to that observed with ascorbic acid (24.29 µg/ml).

Nitric oxide (NO) free radical scavenging activity

Nitric oxide exhibits numerous physiological properties and is also implicated in several pathological states [36].

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Inhibition of nitric oxide radical is also a measure of anti oxidant activity. This method is based on the inhibition of nitric oxide radical generated from sodium nitroprusside in buffered saline and measured by Griess reagent [37]. The antioxidant fractions compete with oxygen to react with nitric oxide and hence reduction in nitrite ions.

Essential oil inhibited NO in a dose-dependent manner with its IC_{50} value being 0.175 mg/ml while ascorbic acid exhibited activity at 25 µg/ml as exhibited in Fig.4. The NO scavenging activity can be used to retard or minimize the damage caused by NO radical and so also its metabolite ONOO- (peroxynitrite) which is extremely reactive and induces toxic reactions including, SH-group oxidation, protein tyrosinase nitration, lipid per oxidation and DNA modifications [38].

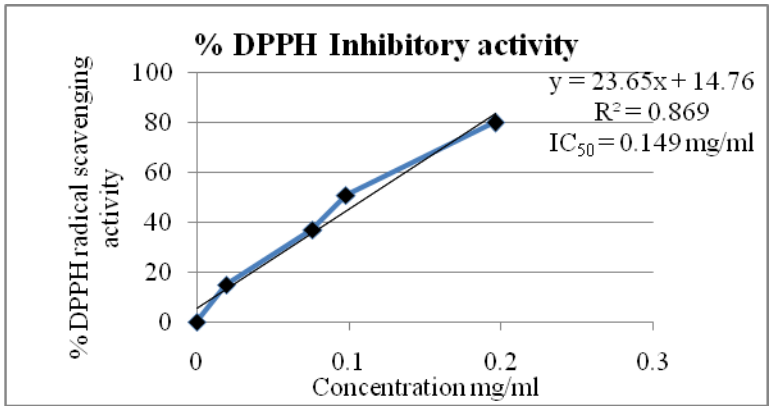

Fig.3. Percentage DPPH inhibitory activity at different concentrations of volatile oil of O. sanctum, L

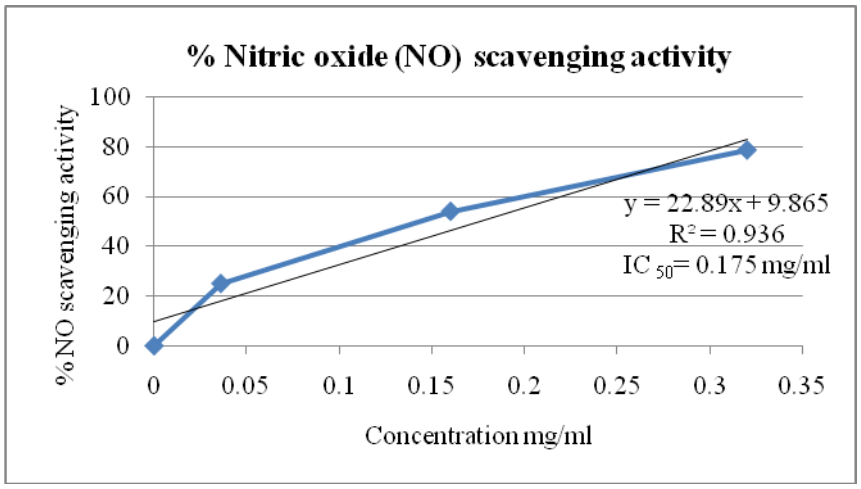

Fig.4. Nitric oxide (NO) scavenging activity at different concentrations of essential oil of O. sanctum, L

ABTS assay

The spectrophotometric technique, the Trolox equivalent antioxidant capacity (TEAC) involves the generation of the long-lived specific radical cation chromophore of 2,2-azinobis-(3-

ethylbenzothiazoline-6-sulphonic acid) (ABTS) by controlled chemical oxidation. This assay is based on interaction between antioxidant and the $ABTS^{\bullet+}$ radical cation which has a characteristic color showing absorption maxima in the near-infrared region at 645, 734 and 815 nm [39]. The TEAC reflects the ability of the electron-donating antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation as compared with that of standard. It is based on the principle of the suppression of the intensely colored (blue-green) $ABTS^{\bullet+}$ by the antioxidant and hence is inversely proportional to the absorbance of the radical cation.

ABTS radicals are more reactive than DPPH radicals and unlike the reactions with DPPH radical which involve H atom transfer; the reactions with ABTS radicals involve electron transfer process.

Using linear regression equation in the standard curve, the TAA of the essential oil was determined as 123 EAC/ µg of sample in the time period of 15 min as in Fig.5.

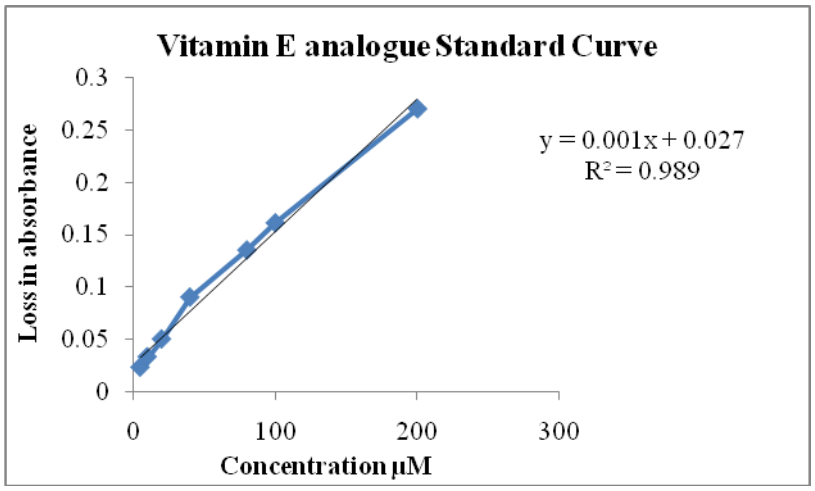

Fig.5. Vitamin E analogue standard curve in ABTS assay

The results of the ABTS assay are expressed in comparison to the standard

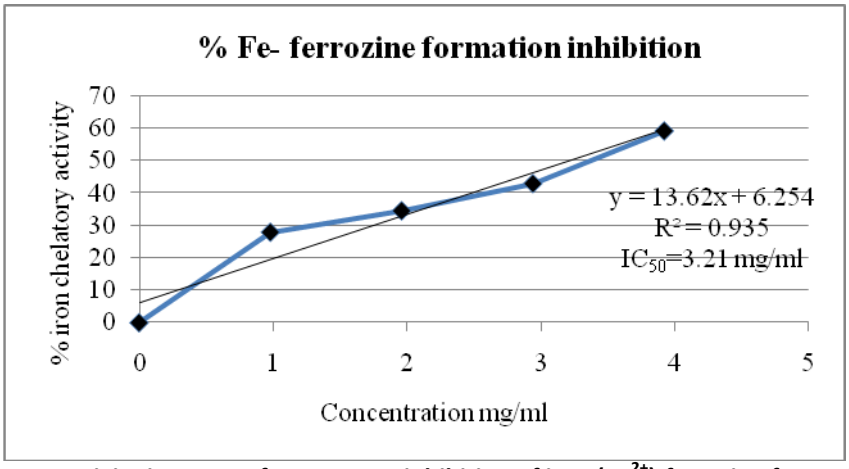

Fig.6. Iron chelatory activity in terms of percentage inhibition of iron (Fe^{2+})-ferrozine formation at different concentrations of volatile oil of O.sanctum, L

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Iron chelating activity

Iron in the reduced form is most important lipid oxidation pro-oxidant & highly reactive amongst all transition metals. Ferrous ion can initiate lipid per oxidation by the Fenton reaction and accelerating per oxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals [40).

Ferrous ion chelation renders important antioxidative effects by retarding metal-catalyzed oxidation. Ferrozine quantitatively form magenta complexes with Fe^{2+}

Fenton Reaction: - Fe^{2+} + ⇨ $Fe^{3+} + H_2O + OH^- + OH^{\cdot}$

In the iron chelating property, ferrozine can make complexes with ferrous ion; in the presence of chelating agents, complex (magenta red) formation is interrupted and as a result the red color of the complex is decreased. The formation of the ferrozine-Fe^{2+} complex is interrupted in the presence of the essential oil indicating the chelating activity with an IC_{50} of 3.21 mg/ml at a much higher concentration than the other assays as shown in Fig.6. EDTA chelated ferrous ion at an activity of 0.017 mg/ml.

Furthermore, chelating agents that forms bonds with a metal are effective as secondary antioxidants since they reduce the redox potential and thereby stabilize the oxidized form of the metal ion [41]. Thus essential oil demonstrated a marked capacity for the iron binding, suggesting their ability as per oxidative protector that relates to the iron chelating capacity.

The free radical theory has stimulated great interest in the role of herbal/dietary antioxidants as therapeutic interventions in many human diseases of polygenic origin including chronic inflammatory ones. This has resulted in designing and developing antioxidant-based therapies for treatment of diseases where no satisfactory therapy is available [42]. The mechanism of antioxidant action in vitro may involve direct inhibition of the generation of ROS, scavenging of free radicals or chelating catalytic metals [43].

From the foregoing discussions, it is clear that not a single method can give a comprehensive prediction of antioxidant efficacy as it involves varied mechanisms. The present study strongly suggests that the essential oil of *Ocimum sanctum*, L have a role as antioxidants by destroying free radicals, scavenging nitric oxide radicals, donating electrons and sequestering iron, avoiding interaction of the metal ion with the membrane lipids thus avoiding oxidative damage to membrane lipids or proteins inhibiting iron-dependent lipid per oxidation.

CONCLUSIONS

The implication of redox mechanisms in the pathogenesis of human diseases and in the process of aging has led to the suggestion that antioxidants in particular, plant diet-derived ones, might have health benefits as prophylactic agents.

In all the testing, a significant correlation existed between concentrations of volatile oil and the polyphenolics with the percentage inhibition of free radicals and metal chelation, indicating a good antioxidant property. Thus, eugenol containing basil plants can have therapeutic implications in neurodegenerative disorders including Alzheimer's or Parkinson's disease, wherein oxidative processes are involved in its etiology by modulating various pathways owing to its strong anti-inflammatory and antioxidant activities.

ACKNOWLEDGEMENTS

The authors like to express their gratitude to UGC-SAP for funding the research project.

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