

In-vitro antioxidant activity of ethanolic extracts of *Centella asiatica L.*, *Oregano vulgare sub.sp hirtum* and *Ocimum basilicum L.* via five model systems

Pooja Adtani¹, N.Malathi^{1*}, D.Chamundeeswari², M.G.Dinesh³

1. Department of Oral Pathology, Faculty of Dental Sciences, Sri Ramachandra University, Chennai

2. College of Pharmacy, Sri Ramachandra University, Chennai

3. Herbal & Indian Medicinal Research Laboratory, Sri Ramachandra University, Chennai

*Corresponding author:Email:narasimhan.malathi@gmail.com

ABSTRACT

Cells undergo oxidative stress under two circumstances either when optimal nutrition is lacking or during diseased conditions. Under such circumstances reactive oxygen species are produced that cause damage to DNA, proteins and cell membrane. Synthetic antioxidants cause several side effects when compared to natural antioxidants. Antioxidants in plants, herbs, spices, fruits and vegetables are present in the form of active constituents namely terpenoids, flavanoids and polyphenols which can play a protective role in inactivating harmful reactive oxygen species. The activity of antioxidants largely depends upon their ability to chelate metal ions or scavenge free radicals. In this study; on comparing the antioxidant activity of Ethanolic extracts of *Centella asiatica L* (EtCA), *Oregano vulgare subsp hirtum* (EtOV) and *Ocimum basilicum L* (EtOB) via five different model systems we observed that EtCA showed maximum activity via Nitric oxide radical scavenging assay and superoxide radical scavenging activity, EtOV via Total Antioxidant assay and Reducing power assay and EtOB via 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay with maximum activity at 1000µg/ml (p<0.01). The results suggest that Ethanolic extracts of *Centella asiatica L.*, *Oregano vulgare subsp hirtum* and *Ocimum basilicum L* exhibited potent antioxidant activity and their active constituents can be further evaluated and studied for formulating a polyherbal antioxidant drug.

Keywords: *Centella asiatica L.*, *Oregano vulgare subsp hirtum* and *Ocimum basilicum L.*, antioxidant

1. INTRODUCTION

Oxidative stress is defined as an imbalance between oxidants and antioxidants, which results in subsequent damage to cell molecules constituting to the production of reactive oxygen species (ROS). ROS such as hydroxyl ions (OH[•]), superoxide radicals (O₂^{•-}), peroxide (ROO[•]) and nitric oxide radicals are produced in living organisms during excessive metabolism; causing several pathophysiological states such as cardiovascular diseases, neurodegenerative diseases, cancer, inflammatory conditions and aging. The most effective way to eliminate and suppress the action of ROS is with the help of antioxidants. Antioxidants both endogenous and exogenous, wether natural or synthetic can prove effective in counteracting free radicals by scavenging or suppressing them.

The use of antioxidants was initiated by ancient Egyptians; they used plants having high content of phenolic compounds to preserve dead bodies (John, 2010). In India plants are being used as a source of traditional medicine since the times of Charaka and Shushruta. Medicinal plants have global importance due to the presence of phytoconstituents with effective pharmacological action. Today several synthetic antioxidants like Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) are available commercially and are being used as

preservatives in processed food. These synthetic antioxidants are carcinogenic and cause several side effects (Ito, 1983). Today natural antioxidants contained in rich amounts in plants, fruits and vegetables have attracted interest due to their nutritional, safety and therapeutic value. Natural antioxidants are present in the form of phenolic compounds in plants (Kahkoven, 1999). Scientific studies in literature have indicated promising phytoconstituents that can be extracted from single or multiple plant extracts for the development of herbal drugs. Nutraceutical formulations are cost effective and have minimum side effects and this is one of the major reasons that the quest for formulating a prime natural antioxidant has become a major scientific research and industrial challenge. Considering the advantages of natural antioxidants over synthetic antioxidants are study aimed at comparing the antioxidant activity of ethanolic extracts of *Centella asiatica Linn*, *Oregano vulgare subsp hirtum* and *Ocimum basilicum Linn* via five different antioxidant assay systems.

Centella asiatica Linn belongs to the ‘Apiaceae or Umbelliferae family’; it is an herbaceous plant native to Srilanka, Malaysia and all over India. In Tamil Nadu it is known as ‘Vallarai Keerai’ and is routinely cooked in households. Other well known names are Pennywort, Mandukparni,

Pegaga and Gotu Kola. It is effectively used in traditional Chinese and Ayurvedic medicine for treatment of ailments such as memory loss, anemia, blood disorders, bronchitis, inflammatory disorders and neurodegenerative diseases. These pharmacological actions are mainly due to the presence of large reservoirs of phenolic compounds and terpenes (Asiaticoside, Asiatic acid, Madecassoside and Madecassic acid). *Oregano vulgare subsp hirtum* commonly known as 'Oregano' is a culinary herb that belongs to the 'Lamiaceae family'. It is cultivated in Europe, North Africa and few parts of India. It is not only used as a spice but also as a medicinal plant for its powerful antioxidant and antimicrobial activity (Adam, 1998). Literature documents that the presence of antioxidant activity is mainly due to the presence of flavanoids and phenolic acids. Oregano had a large pool of polyphenols such as rosmarinic acid and flavanoids like luteolin.

Ocimum basilicum Linn popularly known as 'Sweet basil' also belongs to the Lamiaceae family. It is native to Asian countries one among them being India. It is a popular medicinal plant in Unani and Ayurvedic systems of Medicine. It has a wide spectrum of pharmacological actions such as antioxidant, anti-inflammatory, antimicrobial, anticancer and antiviral (Galila, 2012). Plants of genus *Ocimum* have a large content of phenolic compounds and essential oils which are responsible for its therapeutic actions (Alia Bilal, 2012).

2. MATERIALS AND METHODOLOGY

2.1 Chemicals and reagents: 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and Quercetin was purchased from Sigma - Aldrich. Potassium Ferricyanide, Tricarboxylic acid, Nitroblue Tetrazolium, Phenazine Methosulphate were purchased from Merck (Mumbai, India).

2.2. Plant material: Fresh leaves of *Centella asiatica* Linn, *Oregano vulgare sub ssp hirtum* and *Ocimum basilicum* Linn were collected from Tuticorin, Tamil Nadu, India and were further taxonomically identified and authenticated by Dr.P.Jayaraman, Plant Anatomy Research Centre, Chennai, Tamil Nadu, India.

2.3. Preparation of ethanolic extracts: The leaves after procuring were shade dried and coarsely powdered using a mortar and pestle. 140gms of coarse powder of *Centella asiatica* Linn, 180gms of coarse powder of *Oregano vulgare sub ssp hirtum* and 200gms of *Ocimum basilicum* Linn was subjected to extraction via the maceration technique with 1L ethanol each. The pooled extract was collected after every 24 hours, 48 hours and 72 hours. The pooled

extract was further subjected to a rotary flash to separate the alcohol from the plant extract. The obtained plant extract was further concentrated using water bath. The final extracts were preserved in an air desiccator.

2.4. Determination of secondary metabolites: Preliminary Phytochemical screening was performed to determine the presence of terpenoids, flavanoids and phenols according to Harbone et al

2.4.1. Test for Terpenoids: Noller's Test: The ethanolic extracts were warmed with tin and thionyl chloride. Pink colouration indicated the presence of terpenoids. (Figure B)

2.4.2. Test for Steroidal Terpenoids: Libermann Burchard Test: The ethanolic extract was dissolved in a few drops of chloroform. 3ml of acetic anhydride, 3ml of glacial acetic acid was added, warmed and cooled under the tap. Few drops of concentrated sulphuric acid were added along the sides of the test tube. Appearance of a brown band indicated the presence of steroidal terpenoids. (Figure A)

2.4.3. Test for Flavanoids: The ethanolic extracts were dissolved in alcohol. Drops of 40% Sodium hydroxide solution were added and warmed. Dark yellow coloration indicated the presence of flavonoids. (Figure D)

2.4.4. Test for Phenols: To the ethanolic extracts few drops of alcohol and ferric chloride solution were added. Bluish green coloration indicated the presence of phenols. (Figure C)

2.5. Preparation of extract sample for Antioxidant Assays: 10mg of the extract was dissolved in 1 ml of Dimethyl sulphoxide and 9ml of water and shaken well. The serial dilutions of the extracts (1000µg, 800µg, 400µg, 200µg, 100µg, 50µg, 10µg) were prepared by dissolving the extracts in distilled water.

2.6. Determination of antioxidant activity:

2.6.1. Total antioxidant activity: 0.2ml of various concentration of ethanolic extracts of *Centella asiatica* Linn, *Oregano vulgare subsp hirtum* and *Ocimum basilicum* (1000µg, 800µg, 400µg, 200µg, 100µg, 50µg, 10µg) 2ml of reagent mixture (0.6M of sulfuric acid, 28mM sodium phosphate, 4mM Ammonium molybdate in 1000ml of water) was added and incubated at 95°C for 90 minutes. Distilled water was used as blank. The procedure was repeated thrice and the absorbance was measured using UV spectrophotometer at 695nm. Quercetin was used as positive control. The mean and standard deviation were calculated.

2.6.2. DPPH radical scavenging activity: 0.1ml ethanolic extracts of *Centella asiatica* Linn. *Oregano vulgare subsp hirtium* and *Ocimum basilicum* extract of various concentrations (1000µg, 800µg, 400µg, 200µg, 100µg, 50µg, 10µg) was mixed with 1.9ml of 200µM of DPPH and was allowed to stand for 20minutes for the reaction to occur. The absorbance was determined at 517nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ of inhibition} = \frac{\text{Control} - \text{Test sample}}{\text{Control}} \times 100$$

The percentage inhibitions were plotted against respective concentrations used. Ascorbic acid was used as a positive control. The mean and standard deviation were calculated.

2.6.3. Nitric oxide scavenging activity: 1ml of ethanolic extracts of *Centella asiatica* Linn. *Oregano vulgare subsp hirtium* and *Ocimum basilicum* of various concentration (1000µg, 800µg, 400µg, 200µg, 100µg, 50µg, 10µg) was mixed with 2ml of 10mM of Sodium Nitro prusside in PBS. The mix was then allowed to incubate at 37°C for 4hours. The absorbance of the chromophore was evaluated at 546nm on adding 0.5ml of Griess reagent by the following equation: % of inhibition = $\frac{\text{Control} - \text{Test sample}}{\text{Control}} \times 100$

The percentage inhibitions were plotted against respective concentrations used. Ascorbic acid was used as a positive control. The mean and the standard deviation were calculated.

2.6.4. Reducing power: To 1ml of various concentrations of the ethanolic extracts of *Centella asiatica* Linn. *Oregano vulgare sub ssp hirtium* and *Ocimum basilicum* (1000µg, 800µg, 400µg, 200µg, 100µg, 50µg, 10µg) 2.5ml Phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% Potassium ferricyanide was added and incubated at 50°C for 20 minutes. To the reaction mixture 2.5ml of 10% Tricarboxylic acid was added and centrifuged at 3000rpm for 10 minutes. 2.5ml of supernatant was taken in another test tube to which 2.5ml of distilled water and 0.1% Ferric Chloride was added. Distilled water was used as blank. The procedure was repeated thrice and the

absorbance was measured using UV spectrophotometer at 700 nm. Quercetin was used as a positive control. The mean and standard deviation were calculated.

2.6.5. Superoxide radical scavenging activity assay: The scavenging activity of ethanolic crude extracts to quench superoxide ions was determined according to the suggested methodology of Robak & Gryglewski et al (Robak J, Gryglewski RJ, 1998). To 1ml of various concentrations of the ethanolic extracts of *Centella asiatica* Linn. *Oregano vulgare subsp hirtium* and *Ocimum basilicum* (1000µg, 800µg, 400µg, 200µg, 100µg, 50µg, 10µg), 0.5ml of 16mM of TRIS HCl buffer pH 8, 0.5ml of 0.3mM Nitroblue tetrazolium, 0.5ml of 0.936mM of Nicotinamide Adenine Dinucleotide Reduced. 0.5ml of 0.12mM Phenazine Methosulphate was added and incubated in dark for 5 minutes. Distilled water was used as blank. The procedure was repeated thrice and the absorbance was measured using UV spectrophotometer at 560 nm. % of inhibition was calculated using the formula. % of inhibition = $\frac{\text{Control} - \text{Test sample}}{\text{Control}} \times 100$

Quercetin was used as a positive control. The mean and the standard deviation were calculated.

2.7. Statistical analysis: The assays were performed in triplicates for which mean and standard deviation was calculated by one-way Anova and student 't' test. The antioxidant activity of the three ethanolic extracts was analyzed statistically using SPSS 16.0 version software.

3. RESULTS

The EtCA, EtOV and EtOB showed the presence of Steroidal triterpenoids, terpenoids, flavanoids and polyphenols. EtCA, EtOV and EtOB exhibited increasing antioxidant activity with increasing concentrations in a dose dependent manner; highest at 1000µg/ml. EtCA demonstrated the highest activity via Nitric oxide and Superoxide radical scavenging activity assay. EtOV exhibited via Total antioxidant assay and Reducing power assay; whereas EtOB demonstrated the highest activity via DPPH assay.

Table.1. Phytochemical analysis of *Centella asiatica* Linn, *Oregano vulgare subsp hirtum*, *Ocimum basilicum* Linn

Phytochemical constituents	<i>Centella asiatica</i> Linn	<i>Oregano vulgare subsp hirtum</i>	<i>Ocimum basilicum</i> Linn
Triterpenoids	-	+	+
Steroidal terpenoids	+	-	-
Flavonoids	+	+	+
Polyphenols	+	+	+



Figure.A. Libermann Burchard Test showing a brown ring for the presence of steroidal terpenoids in *Centella asiatica L*

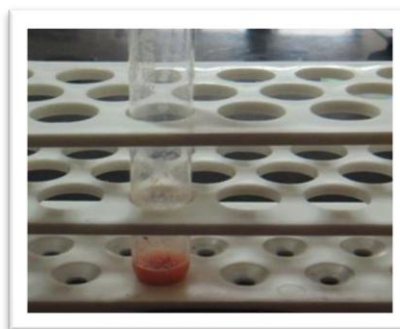


Figure .B. Libermann Burchard Test showing the pink color for the presence of terpenoids in *Oregano vulgare subsp hirtum & Ocimum basilicum L*

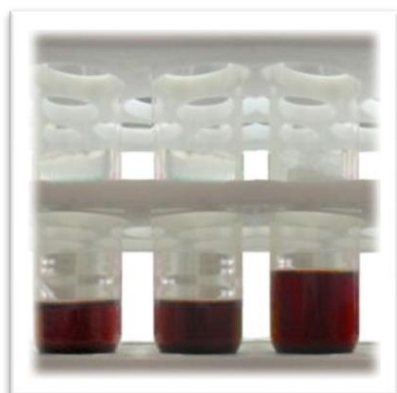


Figure C: Test for Phenols Test showing positivity for polyphenols in *Centella asiatica L (CA), Oregano vulgare subsp hirtum (OV) and Ocimum basilicum L (OB)*

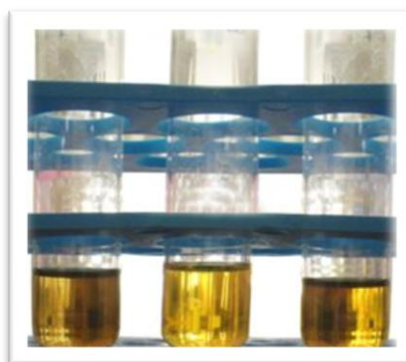
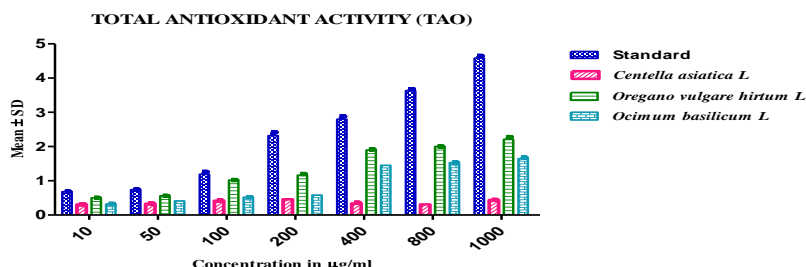


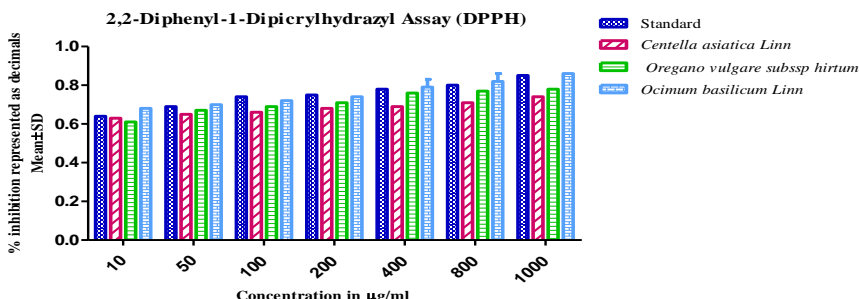
Figure D: Test for flavanoids Test showing positivity for polyphenols in *Centella asiatica L (CA), Oregano vulgare subsp hirtum (OV) and Ocimum basilicum L (OB)*

Figure.1. Total antioxidant activity assay



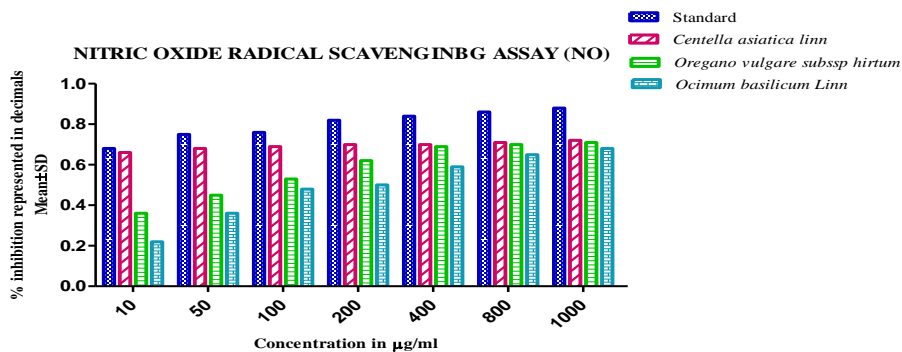
Antioxidant activity of Ethanolic extracts of *Centella asiatica Linn*, *Oregano vulgare subsp hirtum* and *Ocimum basilicum Linn* compared with standard Quercetin. Results are mean values \pm SD of independent experiments performed in triplicate. Results expressed are statistically significant with $** p < 0.001$ by one way ANOVA and student 't' test using Graph Pad prism 5 software.

Figure.2. DPPH assay



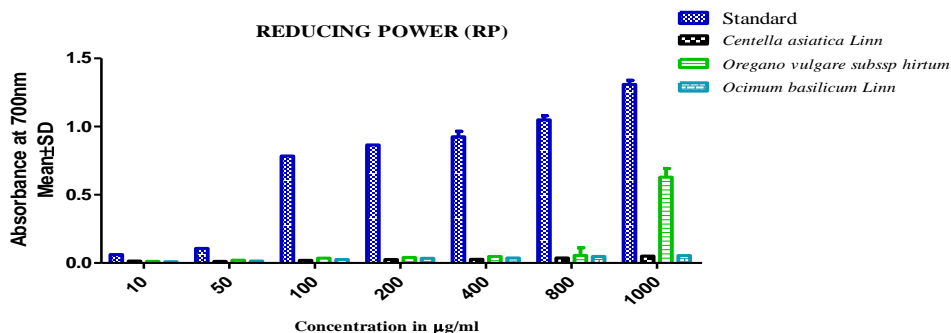
Antioxidant activity of Ethanolic extracts of *Centella asiatica Linn*, *Oregano vulgare subsp hirtum* and *ocimumbasilicum Linn* compared with ascorbic acid. Results are mean values \pm SD of independent experiments performed in triplicate Results are expressed as percentage of inhibition. Observed values are statistically significant with $** p < 0.001$ by one way ANOVA and student 't' test using Graph Pad prism 5 software.

Figure.3. Nitric oxide radical scavenging assay



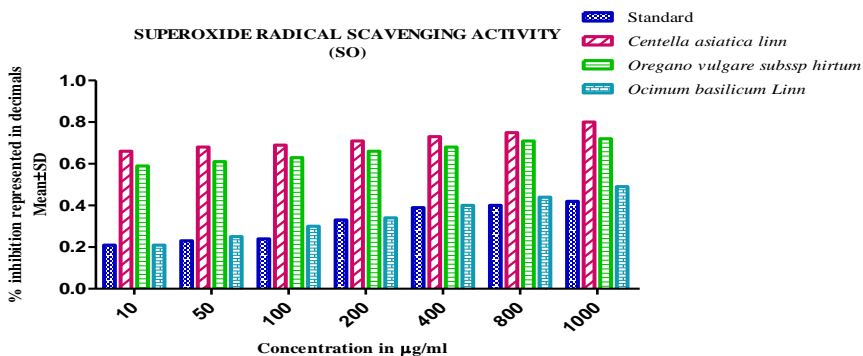
Antioxidant activity of Ethanolic extracts of *Centella asiatica* Linn, *Oregano vulgare subssp hirtum* and *Ocimum basilicum* Linn compared with standard Ascorbic acid. Results are mean values ± SD of independent experiments performed in triplicate. Results are expressed in percentage inhibition which are statistically significant with ** p <0.001 by one way ANOVA and student 't' test using Graph Pad prism 5 software.

Figure 4: Reducing power



Antioxidant activity of Ethanolic extracts of *Centella asiatica* Linn, *Oregano vulgare subssp hirtum* and *Ocimum basilicum* Linn compared with standard Quercetin. Results are mean values ± SD of independent experiments performed in triplicate. Results expressed are statistically significant with ** p <0.001 by one way ANOVA and student 't' test using Graph Pad prism 5 software.

Figure 5: Superoxide Radical Scavenging Assay



Antioxidant activity of Ethanolic extracts of *Centella asiatica* Linn, *Oregano vulgare subssp hirtum* and *Ocimum basilicum* Linn compared with Quercetin. Results are mean values ± SD of independent experiments performed in triplicate. Results are statistically significant with ** p <0.001 by one way ANOVA and student 't' test using Graph Pad prism 5 software.

DISCUSSION

Oxidative stress causes damage to DNA, protein, membrane lipids and cellular organelles leading to the development of cardiovascular diseases, neurodegenerative disorders, cancer and early aging (Pittella, 2009). Maintenance of high intracellular antioxidant level is considered crucial for providing a

reducing environment within the cells to help protect the cell against oxidative damage (Valko, 2006). Several drugs such as steroids, retinoid and synthetic antioxidants such BHT and BHA have been employed for the treatment of ailments but all of these are double edged swords; they help with enormous side effects. To combat this situation today researchers are focusing on developing natural antioxidants which are

easily available, cost effective and have minimum side effects. In our study we observed that all the three ethanolic extracts of *Centella asiatica* Linn, *Oregano vulgare* subsp *hirtum* and *Ocimum basilicum* Linn exhibited potent antioxidant activity in a dose dependent manner with highest activity at 1000µg/ml.

The antioxidant activity of Ethanolic extract of *Centella asiatica* Linn can be attributed to the presence of active constituents such as terpenes such as Madecassic acid, Asiatic acid and three asiaticosides namely asiaticoside, asiaticoside A and asiaticoside B and phenolic constituents predominantly flavanoids and polyphenols. Phenolic compounds are known to have redox properties which help them act as hydrogen donors, reducing agents and singlet oxygen quenchers. In addition they also exhibit potent metal chelation potential. Polyphenols on the other hand have oxidation-reduction properties that play an important role in neutralizing free radicals (Brinkhaus, 2000; Obayed, 2009; Syed Ali Raza, 2009). These properties of active constituents in plants could be responsible for their antioxidant activity.

Similarly; the antioxidant activity of Ethanolic extract of *Oregano vulgare subsp hirtum* can be attributed to the presence of phenolic constituents such as rosmarinic acid, thymol, carvacrol and eugenol which make them singlet oxygen quenchers and hydrogen donors. Flavanoids such as luteolin, herbacetin and quercetin also help in functioning as hydrogen donors (Cervato, 2000; Khanum, 2011). The reducing ability of the extract increased with increasing concentration, our results were in accordance to studies done by Kacaniova et al, Khanum et al and Gulcin et al. Lastly, the antioxidant activity of Ethanolic extract of *Ocimum basilicum* Linn can also be attributed to the presence of terpenoids namely linalool an essential oil which has hydrogen donating ability which helps it to quench reactive oxygen species, polyphenols such as rosmarinic acid an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid that contributes majorly to the plants defence system (Abdullah, 2009). The antioxidant activity of plants may vary depending upon the geographical area, seasonal variation and also on the method and solvent used for extraction.

To the best of our knowledge the evaluation of antioxidant activity of ethanolic extracts of *Centella asiatica* Linn, *Oregano vulgare* subsp *hirtum* and *Ocimum basilicum* Linn and there comparison via five different assay systems has been carried out for the first time.

4. CONCLUSION

With the above results and observations researchers can further probe into the activity exhibited by individual active constituents and extract them through standard procedures for drug formulation. Natural antioxidants in plants, herbs, spices, fruits and vegetables can be used in Nutraceutical application either alone or as an adjunct to treatment modality to combat the disease processes and help in improving the quality of life.

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