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Antimicrobial and Antioxidant Activities in the Root, Stem and Leaf Extracts of *Centella asiatica*

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Abstract

Antimicrobial properties in the root, stem and leaf extracts of medicinally important *Centella asiatica* were determined by studying the activity of chloroform, ethanol and petroleum ether extracts against Escherichia coli, Staphylococcus aureus, Aspergillus niger and Rhizopus stolonifer using MIC method and the antioxidative defence system was evaluated by studying their scavenging capacities against DPPH, O2.-, H2O2 and OH along with the levels of ascorbic acid, reduced glutathione, α-tocopherol and the activities of superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (APX, EC1.11.1.11). The levels of secondary metabolites such as phenols, flavonoids, tannins, steroids, alkaloids were quantified in the root, stem and leaf extracts. The enzyme polyphenol oxidase has been assayed spectrophotometrically and by Native gel analysis. *Centella asiatica* extracts have shown effective, high anti-microbial activity against fungal strains compared with that of bacteria, which might be due to the presence of secondary metabolites. Leaf tissues were found to be possessing high antioxidant activities and secondary metabolites when compared with stem and root tissues. Our results indicate that *Centella asiatica* is a potential source of bioactive compounds responsible for the antimicrobial and antioxidant properties.

Keywords: *Centella asiatica*; Minimum inhibitory concentration; Antioxidants; Polyphenol oxidase

Abbreviations: MIC: Minimum Inhibitory Concentration; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; APX: Ascorbate Peroxidase; PPO: Poly Phenol Oxidase

Introduction

Infections caused by various pathogens are becoming a major threat to public health particularly in developing countries. Usage of improper and synthetic medicines is leading to the development of multi-drug resistant pathogenic strains along with the side effects, enabling to search for the novel compounds with resistance to the emerging new strains. Traditionally, plants are being used as the source for the treatment of various ailments due to the presence of reservoir of chemical agents with therapeutic properties and are the cheapest and safe alternative sources of antimicrobials [1]. Plants are rich sources of various phytochemical molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites with antimicrobial and antioxidant properties [2,3].

Oxidative stress is one of the major factors responsible for the induction of many chronic and degenerative diseases such as atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression etc. [4]. It is a major source for the generation as well as the production of free radicals in foods, drugs and in all aerobic organisms [5]. Reactive oxygen species (ROS) such as superoxide anions O_2,- hydrogen peroxide (H_2O_2), and hydroxyl (OH.) are involved in rendering the oxidative damage to the living systems by the oxidation of lipid, DNA, protein, carbohydrate and other biological molecules. Living organisms possess an antioxidative defence system to eliminate and diminish the ROS generation comprising of enzymatic and non-enzymatic antioxidants. A great number of plants contain certain antioxidants such as ascorbic acid, glutathione, which are known to inhibit lipid peroxidation and ROS generation. There is an upsurge of interest in the therapeutic potentiality of medicinal plants as antioxidants in reducing oxidative stress injury [6].

Centella asiatica popularly known as Brahmi is a prostrate, stoloniferous and perennial herbal creeper belongs to the family of Apiaceae (Umbelliferae) [7,8]. They grow up to a height of 15cm in shady, damp, marshy and wet places. This plant is found throughout the tropical and sub-tropical regions of India. It is used against digestive disorders, skin problems and in wound healing [9]. It is also used to treat asthma, diarrhoea, epilepsy, mental fatigue, stomach ulcers, syphilis and hepatitis. *C. asiatica*

rich in triterpenoids plays an effective role in wound healing by activating the antioxidants at the site of infection. It increases the supply of blood in the affected area and strengthens the skin, thereby enhancing quick healing of the wound [10]. It is also rich in vitamin A, C, B1, B2, niacin and carotene. The medicinal plant *Centella asiatica* is rich in amino acids, essential oils, alkaloids, terpenoids and flavonoids [11]. The whole plant parts are rich in medicinal values. The antimicrobial and antioxidant properties of the leaf extracts were studied well, but not much information is available regarding the antimicrobial and antioxidant properties of its root and stem. In the present study, the antimicrobial and antioxidative properties of root, stem and leaf of *C. asiatica* has been compared to the identification of its novel medicinal properties.

Materials and Methods

Plant material and growth conditions

Centella asiatica plants were propagated in the GITAM University botanical garden in 12 inch pots under 720 minutes natural photoperiod [Irradiance (400-700nm) of 1600-1800µmols m^{-2} s^{-1}] with day/night temperatures of 30 °C /23 °C with an approximate air humidity of 60%. The pots were arranged in rows 1 m apart and the plants were irrigated daily and fertilized weekly with Hoagland solution. Three months old plants with uniform growth were selected for this study.

Antimicrobial activities

Preparation of extracts: Root, stem and leaves of *Centella* were washed thoroughly under running tap water and then with distilled water to remove the dirt and to reduce the microbial load. They were air-dried under shade away from sunlight for 4-5 days, made into a fine powder using mortar and pestle. Extracts were prepared using solvents ethanol, petroleum ether and non-polar solvent chloroform. Leaf, root and stem powder were added to the solvent separately and allowed for the extraction of secondary metabolites with vigorous shaking for 48-72hrs, followed by the filtration and evaporation of solvent using Rota-evaporator resulting in a concentrated extract which can be further diluted to the required concentration in DMSO and is used for assessing their anti-microbial activities by studying Minimum Inhibitory Concentration (MIC) against bacterial and fungal strains.

Antimicrobial studies were carried out with ethanol, petroleum ether and chloroform extracts of *Centella* against two bacterial species of one gram positive strain and one gram negative strains namely *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 1652) and two fungal strains *Aspergillus niger* (MTCC 282) and *Rhizopus stolonifer* (MTCC 2591) obtained from Microbial Type Culture Collection Centre, Institute of Microbial Technology (IMTECH), Chandigarh, India. The glycerol stocks were prepared for both the fungal and bacterial strains, stored at -80°C until further use.

MIC was performed as per clinical and laboratory standard institute guidelines using *Centella* extracts against bacterial and fungal pathogens in a 96 well u-bottomed microtitre plates using p-iodonitrotetrazolium violet as an indicator dye. The anti-microbial compound was serially diluted from the concentration of 500mg to 0.02mg and then added with the final inoculum of 5×10^5 CFU/mL. The anti-microbial compound and the final inoculum were in the ratio of 1:1. After the addition of inoculum, plates were sealed with aluminium foil and incubated at 37 °C for 24hrs in the case of bacterial cultures and for 48hrs at 28 °C for fungal cultures respectively in an incubator. At the end of incubation period, the wells were added with 40µL of 0.2mg/mL p-iodonitrotetrazolium violet dye and incubated for another 30min for the colour development. Presence of bacterial or fungal growth is indicated by a change in the colour of the medium to red, whereas no colour change indicates the absence of growth of the organism and the least concentration where there is no growth is considered as an MIC value of that particular compound against bacterial and fungal strains used. Ampicillin and Fluconazole were used as standards.

ROS scavenging activities

50g dry leaf powder was dissolved in 500mL methanol for 48hrs on orbital shaker at 35 °C with 200 rpm, filtered and concentrated in a rota-evaporator. The concentrated extract was diluted with methanol to obtain IC50 values, concentrations which were used for estimation of scavenging activities of O_2-, OH. and H_2O_2. DPPH free radical scavenging activity of the root, stem and leaf methanolic extracts of *Centella asiatica* were estimated according to Sreejayan & Rao [12]. Superoxide radical scavenging assay was performed according to Yen & Chen [13]. 100µL of the test solution was added to 100µL of 0.1 M phosphate buffer (pH 7.4), 63µL of 468µM NADH, 150µM NBT and 60µM Phenazonium metho sulphate. The Contents were incubated at room temperature for 5min and the absorbance was read at 560nm. Percentage of activity was estimated for 100µg/mL concentration. The Hydroxyl radical scavenging activity of *Centella* was quantified using 2'-deoxyribose oxidative degradation as described by Lopes. The reaction mixture contained 0.2mL of 2.8mM deoxyribose, 0.4mL of 0.2mM ferric chloride, 1.04mM EDTA, 0.2mL of 1mM H_2O_2 and 0.2mL of 1mM ascorbic acid and 5 µL *Centella* extract. The contents were incubated for 1 hour at 37 °C and 1.5mL of 2.8% thio barbituric acid was added and incubated for 20min at 95 °C. After cooling, absorbance was measured at 532nm. The ability of *Centella* leaf extracts to scavenge H_2O_2 was assessed by the method of Ruch et al. [14]. H_2O_2 (40 mM) was prepared in phosphate buffer 0.1 M, pH 7.4. Leaf extracts at the concentration of 10mg/10µL were added to 0.6mL H_2O_2 solution and the final volume was made up to 3mL. The absorbance of the reaction mixture was recorded at 230nm. A Solution containing phosphate buffer without H_2O_2 was considered as blank.

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Estimation of total antioxidant, ascorbic acid, glutathione and α -tocopherol contents

The total antioxidant content in the root, stem and leaves of *Centella* was estimated using an antioxidant assay kit (Cayman Chemical Company) by the ABTS method [15,16]. Ascorbic acid content was determined by extracting 1g of *Centella* tissue in 5mL of 10% TCA and centrifuged at 3,500 rpm for 20min. 0.5mL supernatant was added to 1mL of DTC and incubated at 37 °C for 3hrs. 0.75mL of ice cold 65% H2SO4 was added and incubated at 30 °C for 30min. The absorbance was measured at 520nm. Glutathione reduced (GSH) content was estimated by homogenizing 0.5g of tissue with 2.5mL of 5% TCA and centrifuged at 1000rpm for 10min. 100µL supernatant was made up to 1mL using 0.2M sodium phosphate buffer (pH 8.0) and 2mL of 0.6mM DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) solution was added. After incubation for 10min at 37 °C, absorbance was determined at 412nm [17].

α-tocopherol content in *Centella* tissue was determined according to Rosenberg [18]. 2.5g of the tissue was extracted in 50mL of 0.1N sulphuric acid, allowed to stand overnight and filtered. 1.5mL of filtrate was added to 1.5mL of ethanol and 1.5mL of xylene, mixed well and centrifuged at 5000 rpm for 5min to separate the xylene layer. 1mL of 2, 2 dipyridyl reagent was added and mixed well. Absorbance was read at 460nm. 0.33mL of ferric chloride solution was added to all the tubes and incubated at 37°C for 5min. Absorbance was determined at 520nm and the content of α-tocopherol in the sample was calculated using the formula,

Tocopherols (µg/g) = (Sample A520 - A460/ Standard A520) × 0.29 × 0.15

Extraction of enzymes

All experiments were performed at 4 °C. The roots, stem and leaves were excised, washed and blotted dry. 10g of the tissue was homogenized separately in a mortar and pestle in 50 volumes of extraction buffer containing 100mM Tris-Cl (pH 7.5) containing 5mM DTT, 10mM MgCl2, 1mM EDTA, 5mM magnesium acetate and 1.5% PVP-40. The homogenate was squeezed through four layers of cheese cloth and centrifuged at 10,000rpm for 10min. The protein was precipitated with 75% (w/v) ammonium sulphate, spun at 30,000rpm for 30min and the precipitate was dissolved in 50mM Tris-Hcl (pH 7.8) containing 1mM DTT and 2mM EDTA. The preparation was applied to a column of sephadex G-25, equilibrated with 10mM Tris-Hcl (pH 8.0) which contained 1mM DTT, 10mM NaHCO3, 20mM MgCl2 and 0.2mM NADPH. Elutes were collected and used for enzyme assays.

Enzyme assays

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by the method of Beauchamp and Fridovich [19] as modified by Dhindsa & Matowe [20]. The reaction mixture (1mL) contained: 50mM phosphate buffer (pH 7.8), 0.1mM EDTA, 13mM methionine, 75µM Nitro blue tetrazolium (NBT), 2µM riboflavin and 100µL of the supernatant. Riboflavin was added as the last component and the reaction was initiated by placing the tubes under two 15-W fluorescent lamps. The reaction was terminated after 10min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. Reaction product was measured at 560nm. The volume of the supernatant corresponding to 50% inhibition of the reaction was assigned a value of 1 enzyme unit.

Ascorbate peroxidase (APX, EC1.11.1.11) was spectrophotometrically assayed following a decrease in the absorbance at 265nm according to Chen & Asada [21]. The assay mixture contained: 0.25M ascorbate and 1mM H2O2 in 50mM phosphate buffer (pH 7.0) with 37.5µL of the enzyme extract. Corrections were made for the low rates of ascorbate disappearance due to non-enzymatic and H2O2 independent oxidation. Rate of ascorbate disappearance was determined during the linear phase of the reaction. Polyphenol oxidase (PPO, EC 1.10.3.1) activity for both the enzymes catechol oxidase and laccase is assayed by using a spectrophotometer maintained at a wavelength of 495nm. The change in the absorbance is noted for every 30 seconds up to 5min by adding 0.2mL of enzyme extract to 2.5mL of 0.1M Phosphate buffer (pH-6.5) and 0.3mL of 0.01M catechol solution in a cuvette. Polyphenol oxidase activity is calculated by using the formula [22].

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

Where, K for Catechol oxidase=0.272; K for Laccase= 0.242.

Quantitative estimation of secondary metabolites

Estimation of phenols, Flavonoids and Tannins: Total Phenols estimated spectrophotometrically using Folin-Ciocalteau reagent which gives a blue colour complex measured at 650nm with Gallic acid as a standard [23]. 0.5g *Centella* leaf, root and stem samples were homogenized in 80% ethanol and centrifuged for 20min at 10,000rpm. To 3mL of each extract, 0.5mL of FC reagent and 2mL of 20% sodium carbonate was added and absorbance of the samples was measured at 650nm. Flavonoids were estimated according to Chang et al. [24]. 500mg of leaf, root and stem powder was added separately to 5mL of 80% methanol and extracted for 48hrs by shaking at room temperature followed by centrifugation at 10,000rpm for 20min. To 0.5mL of extract, 1.5mL of methanol, 0.1mL of 10% aluminium chloride, 0.1mL of 1M potassium acetate and 2.8mL of distilled water were added and incubated at room temperature for 30min. Absorbance of the samples measured in the wavelength of 415nm using Quercitin as a standard. The content of tannins in *Centella* leaf, root and stem samples was estimated according to Polshettiwar et al. [25] by the addition of 500mg of dried powder to 25mL of distilled water and incubating at 100 °C for 30min and then centrifuged at 10,000 rpm for 20min. To

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1mL of extract, 1mL of Folin-Denis reagent and 2mL of sodium carbonate solutions were added and the volume was made up to 5mL with distilled water, incubated at room temperature for 30min and the absorbance was measured at 700nm. Tannic acid was used as a standard for the preparation of calibration curve.

Estimation of terpenes, anthraquinones and alkaloids

Terpenes were estimated spectrophotometrically by adding 10mL of petroleum ether to 1g of leaf, root and stem powder and extracted with shaking for 15min. The extracts were filtered separately and the absorbance was measured at 420nm [26]. Anthraquinone content in the leaves, root and stem samples of *Centella* was determined by adding 50mg of dried tissue in 50mL of distilled water extracted by shaking for 16h. The contents were incubated at 70 °C and 50mL of 50% methanol was added and filtered. Absorbance of the filtrate was measured at 450nm. Calibration standards were prepared using alizarin and purpurin at a concentration of 0.01mg/1mL [27].

Alkaloid content was estimated by adding 100mg of dried *Centella* powder to 40mL of 95% ethanol refluxed for about half an hour and then filtered. The volume of the filtrate was adjusted to 50mL with 95% ethanol and subjected to evaporation. The residue obtained was treated with 3mL of 1N Hcl and allowed to stand for 2hr hydrolysis. 3mL of 1N NaOH was added, followed by the 2mL concentrated acetic acid and the volume being adjusted to 10mL with distilled water. 1mL of this solution was made up to 5mL with 20% acetic acid and added with 5mL of acetate buffer, 1mL of 0.05% methyl orange and 5mL chloroform. After few minutes chloroform layers are withdrawn, added with a pinch of Na2SO4 and the absorbance was measured at 420nm. Solasodine was used as standard for calibration [28].

Estimation of steroids, saponins, cardiac glycosides and lignins

Estimation of steroids was done by adding 2mL of 4N H_2SO_4, 2mL of 0.5% $FeCl_3$ and 0.5mL of 0.5% potassium hexa cyanoferrate to 1mL of methanolic extract. The contents were incubated at 70 °C for 30min, allowed to cool and made up to the volume of 10mL with distilled water. Absorbance of the samples was measured at 780nm [29]. Saponins were estimated according to Brunner [30]. 1g of fine powdered sample was weighed accurately and added to 100mL of isobutyl alcohol and extracted with shaking for 5hrs and then filtered. To the filtrate, 20mL of 40% saturated magnesium carbonate solution was added and again subjected to filtration through a filter paper; a clear colourless filtrate was obtained. To 1mL of the filtrate, 2mL of 5% $FeCl_3$ solution was added and the volume made up to 50mL with distilled water. The contents were allowed to stand for 30min at room temperature to develop a deep red colour and the absorbance of the samples was measured at 380nm. A calibration curve was prepared using Dioxgenin concentrations ranging from 0-100µg.

For estimation of c Cardiac glycosides, 1g of leaf, root and stem tissue powder was added to 10mL of 70% alcohol and extracted for 2-3h followed by the filtration. 4mL of the filtrate was added to 5mL of 12.5% lead acetate and the volume made up to 50mL with distilled water. The solution was again filtered and 5mL of 4.77% disodium hydrogen orthophosphate was added to 25mL of filtrate resulting in the formation of precipitate removed by a third round of filtration. A 5mL of freshly prepared Buljet's reagent was added to 5mL of clear solution obtained after filtration and incubated at room temperature for 1hr. The absorbance of the samples was measured at 595nm and the calibration curve was prepared using 0.02% Digitoxin dissolved in chloroform-methanol at the ratio of 1:1 [31]. Estimation of lignins was done according to Kent et al. [32] by adding 1mL of 72% sulphuric acid to 0.5g of dry leaf, stem and root powder, incubated at 30 °C for 1hr with occasional stirring. 28mL of distilled water was added and the beaker was incubated at 120 °C for 1hr. The contents were filtered and the residue obtained after filtration was dried overnight at 105 °C and determined the weight (AIR) whereas the filtrate obtained measured at 205nm (ASL).

Acid-insoluble residue (AIR)

Where, m=weight of the residue after drying and M=Oven dry weight of the sample before acid hydrolysis.

Acid-soluble lignin (ASL)

Where, A=Absorbance, D=Dilution factor, V=Volume of the filtrate, a=Extinction co-efficient of lignin, b=Cuvette path length and M=Oven dry weight of the sample before acid addition. Total Lignin Content = AIR+ASL.

Statistical analysis

Results mentioned are reported as the mean±standard error (SE) values of five independent experiments, conducted on five different plants in each experiment. SE values were calculated directly from the data according to standard methods [33]. Data analysis was carried out using the SPSS package. Mean values were compared by Duncan's multiple range test and P-values which are less or equal to 0.05 were considered as statistically significant.

Results and Discussion

Antimicrobial activity

Table 1: Anti-microbial activity of *Centella asiatica* leaf, stem and root extracts against microbial strains.

Strains Used	Leaf C.E (mg/mL)	Leaf E.E (mg/mL)	Leaf P.E (mg/mL)
E. coli	62.5	7.8	125
S .aureus	125	31.25	125
A. niger	1.9	0.48	1.9
R. stolonifer	0.97	15.62	62.5
Strains used	**Stem C.E (mg/mL)**	**Stem E.E (mg/mL)**	**Stem P.E (mg/mL)**
E. coli	125	125	125
S .aureus	125	125	62.5

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A. niger	7.8	1.9	15.62
R. stolonifer	125	62.5	62.5
Strains used	**Root C.E (mg/mL)**	**Root E.E (mg/mL)**	**Root P.E (mg/mL)**
E. coli	125	125	125
S .aureus	62.5	62.5	125
A. niger	31.25	15.62	15.62
R. stolonifer	250	62.5	62.5

In the present study, the ethanol, chloroform and petroleum ether extracts of *Centella asiatica* root, stem and leaf were investigated for antibacterial and antifungal activity against *E. coli*, *S. aureus*, *A. niger* and *R. stolonifer* (Table 1). All the extracts exhibited the antimicrobial activities against the tested bacterial and fungal strains. Among the three extracts used in the present study, *Centella asiatica* plant ethanol extracts showed higher inhibition compared with that of other two extracts. The stem and root extracts of *Centella* were effective against the strains *S. aureus* and *A. niger* compared with other two strains. The leaf ethanol extracts gave effective results inhibiting microbial growth at a concentration of 7.8mg/mL against *E. coli*, 31.25mg/mL against *S. aureus*, 0.48mg/mL against *A. niger* and 15.625mg/mL against *R. stolonifer* as compared to the standards ampicillin and fluconazole, which showed inhibition at a concentration of 125mg/mL against both *E. coli* and *S. aureus* and 3.9, 125mg/mL against *A. niger* and respectively (Table 1). The emergence of various multi drug resistant microbes is the main reason for the increase in the infectious diseases among human beings. Food spoilage due to the contamination of water, milk and other food products by microbes is also considered as the main factor responsible for the serious illness to the human community, forcing to search for the alternative sources of medicines to cure the illness. Medicinal plants are the rich sources of bioactive compounds with antioxidant and antimicrobial properties, which can be used as curative drugs with minimizing the side effects of synthetic drug and provide better adaptability with an economical affordability [34]. All the plant cells are rich in bioactive compounds, but the concentration of each compound varies from one part to the other part of the plant reported by Chanda & Kaneria [35]. Among the different parts of the plant, leaf is considered to be one of the highest accumulator regions for compounds used generally for therapeutic needs [36]. In the present study, the antimicrobial and antioxidant properties of the root, stem and leaf of medicinally important *Centella asiatica* were compared for better understanding of its medicinal properties.

Medicinal plants enhance the knowledge about the relationship between a compound and its biological effects, which can be used as composite natural sources of anti-microbial and anti-infectious compounds. Leaf ethanol extract of *C. asiatica* showed a maximum inhibitory effect on microbes than other extracts which could be due to the presence of the higher percentage of bioactive compounds in its leaves, which can be attributed with low antimicrobial activity in the root extracts. The foliar petroleum ether and ethanol extracts of *Centella* exhibited higher rates of antifungal activity against Candida albicans, Aspergillus niger and Aspergillus flavus compared with water extracts [37]. Several studies reported that the plants rich in bioactive compounds such as tannins, saponins, flavonoids, alkaloids and phenolic compounds possess high antimicrobial properties [38]. The action of bioactive compounds on microbes might be due to the interference of bacterial cell wall peptidoglycan biosynthesis and by inhibiting protein synthesis, nucleic acid synthesis, act as chelating agents, inhibiting the metabolic pathway, disrupting the peptide bonds and preventing the microbes to utilize the available nutrients.

Antioxidant activity

Table 2: DPPH, O_2.-, H_2O_2 and .OH scavenging activities in the root, stem and leaf extracts of *Centella asiatica*.

Radical	Root	Stem	Leaf
DPPH (µg $^{-1}$ mL)	22.9±1.3	31.6±1.4	40.2±1.3
O2.- (µg $^{-1}$ mL)	26.8±1.6	33.9±1.8	45.4±1.5
H2O2 (µg $^{-1}$ mL)	25.6±1.4	29.5±1.6	35.5±1.2
OH (µg $^{-1}$ mL)	8.4±1.5	14.2±1.3	20.6±1.5

Each point is an average of five independent determinations ±SE, (t (4) = 1.2, P < 0.05) for DPPH, ($t_{(4)}$ = 0.8, P < 0.05) for O2.-, ($t_{(4)}$ = 1.5, P < 0.05) for H2O2 and ($t_{(4)}$ = 0.8, P < 0.05) respectively.

Ethanol extracts of *Centella asiatica* root, stem and leaf were tested for their scavenging activities. Foliar extracts of *Centella asiatica* have shown high DPPH (40.2µg-1 mL), O_2- (45.4g-1 mL), H2O2 (35.5g^{-1} mL) and OH. (20.6µg^{-1}mL) scavenging activities compared with that of root and stem extracts (Table 2). Total antioxidant activity was high in the leaves of *Centella asiatica* (8.6mM g^{-1} fw) along with the ascorbic acid (0.8mg g^{-1} fw), reduced glutathione (35.6mg g^{-1} fw) and α- tocopherol (1.1mg g^{-1} fw) (Table 3). The activities of enzymatic antioxidants superoxide dismutase and ascorbate peroxidase were also high in the leaves of *Centella* when compared with that of stem and root (Figure 1&2). ROS comprising various forms of activated oxygen and non-free radicals such as superoxide ions (O^2-), hydrogen peroxide (H^2O^2) and hydroxyl radicals (OH) play an important role in the degenerative or pathological processes oriented with human health necessitating additional defenses [39]. Living organisms are equipped with the antioxidant defense systems, which protects the cells against oxidative damage by scavenging free radicals, chelating free catalytic metals and by acting as electron donors [40]. With the decreasing quality of dietary intake, the amount of the antioxidants synthesized in the body might not be sufficient demanding the dietary intake of antioxidant compounds from medicinal plants due to their non-toxic nature and their multiple mechanisms of actions. The ROS scavenging activities of root, stem and leaf *Centella* extracts were studied by subjecting the ethanolic extracts to DPPH, O_2-, H_2O_2 and OH.

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Table 3: Total antioxidant, Ascorbic acid, reduced glutathione and α-tocopherol contents in the root, stem and leaf extracts of C. asiatica.

Non-enzymatic antioxidants	Root	Stem	Leaf
TEAC content (mM g^{-1} fw)	3.2± 0.05	5.5± 0.2	8.6± 0.7
Ascorbic acid (mg g^{-1} fw)	0.2±0.03	0.4±0.02	0.8±0.02
Glutathione reduced (mg g^{-1} fw)	18.8±1.8	24.2±2.1	35.6±1.2
α-tocopherol (mg g^{-1} fw)	0.6±0.04	0.9±0.03	1.1±0.0.2

Each point is an average of five independent determinations±SE, (t (4) = 0.6, P < 0.05) for TEAC, (t (4) = 1.0, P < 0.05) for ascorbic acid, (t (4) = 0.6, P < 0.05) for GSSG and for α-tocopherol (t (4) = 0.2, P < 0.05) respectively.

Scavenging activities (Table 2). Leaf extracts were shown to possess high ROS scavenging activities compared with root and stem extracts. Further the non-enzymatic antioxidants such as ascorbic acid, reduced glutathione and α-tocopherol were quantified along with the total antioxidant contents in the root, stem and leaves of C. asiatica. Ascorbic acid is one of the most abundant and powerful antioxidant essential forminimizing the ROS damage and ensuring the normal function of the living cells and many enzymatic reactions [41]. Reduced glutathione functions as antioxidant directly by scavenging the ROS or by reducing the oxidized ascorbic acid due its interactions with the ROS. α-tocopherol acts as inter as well as intracellular physiological antioxidant in maintaining the homeostasis of labile metabolites of the cell. It protects oxidizable vitamins, unsaturated fatty acids and maintains cell membrane integrity.

Removal of the ROS leads to the oxidization of α -tocopherol, which will be reduced back to its active form through the reduction by other antioxidants such as AA [42]. Quantification of non-enzymatic antioxidants in the root, stem and leaf tissues have shown that the levels of these three antioxidants were higher in the leaf tissues of *C. asiatica* (Table 3). Enzymatic ROS scavenging mechanisms in plants include superoxide dismutase, which catalyses the conversion of O_2- radicals into H_2O_2 and H_2O, which will be further decomposed by the activity of catalases and ascorbate peroxidase along with ascorbate and glutathione whose co-ordination will eliminate the toxicity of ROS in plant cells [43]. The activities of SOD and APX were assayed in the root, stem and leaves of *C. asiatica* (Figure 1&2), which were higher in the leaf tissues compared with stem and roots, demonstrating that the leaves, stem and even roots of *C. asiatica* are rich in antioxidants, which can be used as an effective protective agents against oxidative stress and various diseases occurs due to the activity of ROS. Leaves of *Abutilon Indicum* have shown the band of 36 kDa SOD protein and a high activity of 2.84 Kat mg^{-1} proteinmin^{-1} [44]. Significant levels of enzymatic and non-enzymatic antioxidants in the leaves of asevolvulusalsinoides showed that its leaves can be used as an effective source of antioxidants [45].

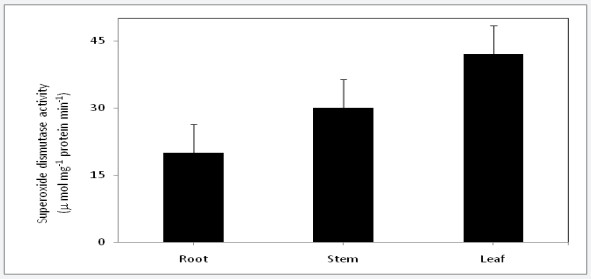

Figure 1: Superoxide dismutase activity in the root stem and leaf extracts of *Centella asiatica*. Each point is an average of five independent determinations±SE, (t (4) = 1.4, P < 0.05).

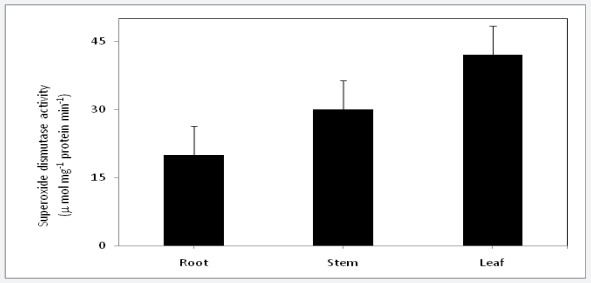

Figure 2: Ascorbate peroxidase activity in the root stem and leaf extracts of *Centella asiatica*. Each point is an average of five independent determinations±SE, ($t_{(4)}$ = 1.4, P < 0.05).

Quantification of secondary metabolites

Quantification of secondary metabolites in the root, stem and foliar tissues of *Centella* has revealed the presence of various bioactive compounds at varying concentrations (Table 4). The concentrations of these compounds were high in the leaves than that of stem and root. However, the concentrations of saponins, lignins and terpenes were high in the root, stem and leaves of *Centella*. High medicinal, antimicrobial and antioxidant properties of *Centella* might also be due to the presence of bioactive compounds.

Medicinal plants are the most exclusive sources of secondary metabolites which are being commercialized as the lifesaving drugs to the majority of the population [46]. Utilization of the plant compounds is gaining attention due to their low side effects. Triterpene saponin Asiaticoside of *Centella* leaves is utilized commercially as a potent anti-inflammatory agent and in anti-gastric ulcers drugs [47]. In the present study, the concentrations of 10 different secondary metabolites were assessed in the root, stem and leaves of *C. asiatica*, which showed the high concentrations of alkaloids, saponins, lignins, terpenes and tannins in its leaves (Table 4). Alkaloids are one of the largest class of plant secondary metabolites which are proficient and significant pharmaco-therapeutic substances possessing anti-inflammatory properties. Phenolics possess a maximum degree of antioxidant properties and are the largest group of phytochemicals. Saponins are responsible for the antimicrobial, hypotensive and cardio depressant properties used in the

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treatment of cardiac myopathy and congestive heart failure [48]. Tannins play a vital role as free radical scavengers.

Table 4: Secondary metabolite concentrations in the root, stem and leaf extracts of *Centella asiatica*.

Secondary metabolite	Root	Stem	Leaf
Tannins ($mg^{-1}g$ dw)	1.40±0.03	2.40±0.04	3.20±0.03
Phenols ($mg^{-1}g$ fw)	1.75±0.06	2.23±0.0.8	2.98±0.05
Flavonoids ($mg^{-1}g$ dw)	1.18±0.04	1.58±0.06	2.56±0.02
Saponins ($mg^{-1}g$ dw)	4.79±0.08	4.85±0.03	5.87±0.05
Anthraquinones ($mg^{-1}g$ dw)	0.82±0.02	1.40± 0.06	2.10±0.08
Lignins (%)	18.1±1.12	22.1± 1.18	29.5±1.25
Terpenes ($mg^{-1}g$ dw)	1.07±0.08	2.33±0.05	3.61±0.09
Steroids ($mg^{-1}g$ dw)	0.98± 0.02	1.94±0.06	2.51±0.12
Alkaloids ($mg^{-1}g$ dw)	0.92±0.03	1.22±0.04	1.98±0.04
Cardiac glycosides ($mg^{-1}g$ dw)	0.54±0.02	0.93±0.03	1.68±0.06

Each point is an average of five independent determinations ± SE, Tannins [t(4)=0.8, P<0.05], Phenols [t(4)=1.1, P<0.05], Flavonoids [t(4)=0.7, P<0.05], Saponins [t(4)=3.2, P<0.05], Anthraquinones [t(4)=0.6, P<0.05], Lignins [t(4)=4.6, P<0.05], Terpenes [t(4)=0.8, P<0.05], Steroids [t(4)=0.2, P<0.05] and Alkaloids [t(4)=0.2, P<0.05].

They also inhibit the microbial growth by binding to the carbohydrate and protein molecules. Presence of terpenoids and steroids causes analgesic activity [49]. The activity of polyphenol oxidase was assayed in the root, stem and leaf extracts of *Centella*, which showed high activity in the leaf (0.816 and 0.726 Units $g^{-1}h^{-1}$ of catechol oxidase and laccase respectively). Enzyme polyphenol oxidase (PPO) has been assayed in the root, stem and leaves of *C. asiatica* (Figure 3), which showed an activity of 0.816, 0.792 and 0.345 units g^{-1} h^{-1} respectively. PPO is one of the widely distributed group of copper enzymes that catalyses the oxidation of phenolic compounds using molecular oxygen as co-substrate [50,51]. When the plant cell gets damaged due to crushing or decay, PPO interacts with the phenolic substrates leads to browning. A melanin pigment forms in the living tissues as a result of defence mechanism protecting from insect attack and microbial contamination.

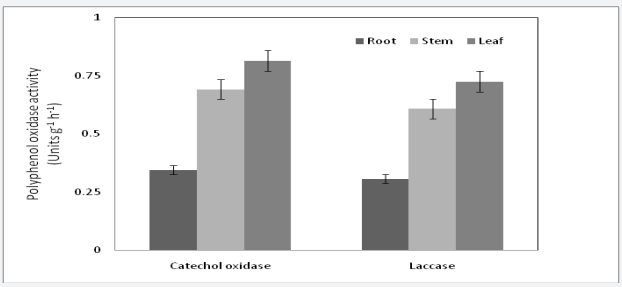

Figure 3: Polyphenol oxidase activity in the root stem and leaf extracts of *Centella asiatica*. Each point is an average of five independent determinations±SE, ($t_{(4)}$ = 0.08, P < 0.05).

Conclusion

It can be concluded that the medicinal plant *Centella* possessing the antimicrobial and antioxidant property can be used in the pharmaceutical research for the development of novel and potential drugs for curing the diseases. In the present study, we have also found the presence of many secondary metabolites which are biologically active in leaf, stem and root samples of *Centella*. All the extracts of *Centella* showed effective inhibitory activity against the tested strains with slight variations. This information regarding *Centella* provides a scope to study the functional properties of bioactive compounds and to use them in an effective manner for the discovery of potential chemotherapeutic agents.

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