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IN VITRO ANTIOXIDANTS STATUS IN SELECTED INDIAN MEDICINAL PLANTS

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ABSTRACT

Five Indian medicinal plants namely *Acorus calamus* L, *Pelargonium graveolens* L, *Cymbopogon martini* L, *Cyambopogon nardus* L and *Cymbopogon citratus* L. were studied for their enzymatic and non enzymatic free radical scavenging properties. Enzymatic ROS scavengers: superoxide dismutase (SOD) and catalase were observed maximum respectively in *A. calamus* (3.59±0.22 µ mol/min/mg/protein) and *P. graveolens* (1195.69±0.012 µ mol/min/mg/protein). However, *C. citratus* which exhibited lowest SOD activity (0.172±0.022), displayed highest activity of ascorbate peroxidase (2.566±0.254 µ mol/min/mg/protein). The ascorbic acid was found to be highest in *C. martini* (592.09±0.0961 mg/100g) whereas carotenoids were observed maximum in *A. calamus* (174.8±3.930mg/100g) plant leaves. The total phenolic content (Folin-Ciocalteu assay) was shown to be highest in *P. graveolens* leaves (22.27±0.284mg GAE ml^{-1}) followed by *C. citrates*, *C. martini*, *C. nardus* and *A. calamus*. The findings indicated promising free radical scavenging activity of the above plants and needs further exploration for their effective use in medicines.

KEY WORDS: Ascorbic acid, Carotenoids, Reactive Oxygen species, Superoxide dismutase, Total phenol.

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INTRODUCTION

Nature has been a source of medicinal agents since times immemorial. The importance of herbs in the management of human ailments cannot be over emphasized. It is clear that the plant kingdom harbors an inexhaustible source of active ingredients invaluable in the management of many intractable diseases. Ayurveda is ancient health care system and is practised widely in India, Srilanka and other countries[1]. Ayurveda system of medicine, use plants to cure the ailments and diseases. Despite the availability of different approaches for the discovery of therapeutically, natural products still remain as one of the best reservoir of new structural types. They are used directly as therapeutic agents, as well as starting material for the synthesis of drugs or as models for pharmacologically active compounds[2]. Aromatic and Medicinal plants are the sources of natural antioxidants, and some of the compounds have significant anti-oxidative properties and health benefits[3]. Antioxidants are a type of complex compounds found in aromatic, medicinal and other plants that act as a protective shield for our body against certain diseases such as arterial, cardiac diseases, arthritis, cataracts and also premature ageing along with several chronic diseases. Reactive oxygen species (ROS), including hydroxyl radicals ($OH^{\cdot}$), superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), lead to lipid peroxidation and specific oxidation of some enzymes and protein oxidation and degradation[4]. ROS generation through normal cellular metabolism and by means of exogenous insult is a constant problem for which cells have developed multiple protective mechanisms to survive[5]. Antioxidants are capable of exerting protective effects against oxidative stress in biological systems[6]. They terminate ROS chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Antioxidant based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer's disease and cancer have appeared during last three decades[7]. In current herbal drug scenario, plant derived antioxidants are gaining importance because of their potential health benefits, no toxicity and side effects over synthetic antioxidants like butyl hydroxy anisole and butyl hydroxy toluene (BHA and BHT, respectively). Plants may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity[8]. The wide array of enzymatic and non-enzymatic antioxidant defenses includes superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), Ascorbate peroxidase (APX) ascorbic acid (vitamin C), α-tocopherol (vitamin E), reduced glutathione (GSH), β-carotene, and vitamin A[9, 10]. The function of antioxidative enzymes is to protect cells from toxic oxygen. Thus, our present studies focus on five medicinal plants namely *Acorus calamus* L*, Pelargonium graveolens* L, *Cymbopogon martini* L, *Cymbopogon nardus* L and *Cymbopogon citratus* L to determine their antioxidant and non enzymatic free radical scavenging properties.

MATERIALS AND METHODS

Collection of medicinal plants

The five medicinal plants were collected from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India and were grown in the green house of the College of Forestry, Sam Higginbottom Institute of Agriculture, Technology & Sciences, Allahabad U.P. The plant materials for analysis were cleaned and powdered. The botanical names, family names, English

names and parts used are presented in Table 1.

Table 1
Brief introduction of medicinal plants.

Botanical Name	English Name	Parts used	Family	Medicinal use
Acorus calamus	Bach	Leaf	Araceae	Brain tonic, Fever, Asthma digestive problems
Pelargonium graveolens	Geranium	Leaf	Geraniaceae	Cough, Kidney pain, gastrointestinal ailments
Cymbopogon martini	Palmarosa	Leaf	Poaceae	Skin disease, treatment of baldness
Cymbopogon nardus	Citronella	Leaf	Poaceae	Muscular pain, Nervous disorder
Cymbopogon citratus	Lemongrass	Leaf	Poaceae	Insect repellent, digestive problems

Estimation of Chlorophyll

The procedure followed was given by Arnon and Stout[11]. Briefly, one gram of leaves was homogenized in 5 ml of 80% acetone (acetone: water, v/v). Extraction was done then cooled. The suspension was centrifuged at 5000 rpm for five min there after supernatant was used for measuring chlorophyll content. The absorbance was recorded at 665 and 663 nm. 80% acetone was used as blank.

Estimation of Protein

Protein content in the plant extracts was determined according to Lowry et al[12]. One gram fresh leaves were homogenized with 10 ml phosphate buffer (1mM, pH 7.0). The homogenate was centrifuged at 8000 rpm for 30 minutes. The supernatant was used for protein estimation. Its 100 µl, and 200 µl of the aliquots were taken in triplicate for test and maintain to 500 µl by water, followed by the addition of 5 ml of reagent-C, (reagent-C: 95 ml of reagent-A mixed with 5 ml of reagent-B, Reagent-A: 2% sodium carbonate in 0.1 M NaOH, Reagent-B: 1% copper sulphate ($CuSO_4.5H_2O$). 2% potassium-sodium tartarate in ratio of 1:1 was also mixed properly and incubated for 10 min at room temperature. 500 µl of 1N Folin-Ciocalteu'sphenol reagent was mixed and vertexed quickly. This reaction mixture was incubated for 30 minutes at 37°C and its absorbance was recorded at λmax 660 nm. The amount of protein was calculated by comparison with standard curve drawn under identical experimental conditions.

Assay of enzymatic antioxidants

Preparation of enzyme aliquot

Approximately one gram preserved samples were homogenized in 10 ml of solution containing 50 mM PBS (pH 7.6) and 0.1 mM Na-EDTA and then centrifuged (Remi 815) for 15 min at 20,000 rpm at 4°C.

Superoxide dismutase (SOD)

For the assay of SOD, 6.0 ml of reaction mixture was prepared in glass vials by adding the constituents in the following order; phosphate buffer (50 mM, pH 7.6), 0.1 mM Na-EDTA, enzyme aliquots (50-150 µM), 50 mM Na_2CO_3 (pH 10.2), 12 mM L-methionine, 75 µM nitro blue tetrazolium chloride (NBT) and at last 2 µM riboflavin was added. The glass vials were kept under a light intensity of about 400 µM m^{-2} s^{-1} in normal room temperature to carry out the reaction. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction measured at 560 nm[13].

Catalase (CAT)

Activity of catalase (CAT) was measured as described by Cakmak and Marschner[13]. The assay was based on the degradation of H_2O_2 at 240 nm. The reaction mixture (1.0 ml) was prepared by adding 0.1 ml of 50 mM PBS (pH 7.6), 0.1 ml of 0.1 mM EDTA, 0.1 ml of 100 mM

H_2O_2 and 0.7 ml of enzyme aliquot. The degradation of H_2O_2 was monitored at 240 nm.

Ascorbate peroxidase (APX)

Activity of APX was measured according to Cakmak[14] by monitoring the rate of H_2O_2-dependent oxidation of ascorbic acid (AsA) at 290 nm. A reaction mixture (1 ml) was prepared containing 0.1 ml of 50 mM of PBS (pH 7.6), 0.1 ml of 0.1 mM EDTA, 0.1 ml of 12 mM H_2O_2, 0.1 ml of 0.25 mM AsA and 0.6 ml of enzyme aliquot.

Assay of non-enzymatic antioxidants

Ascorbic acid (Vit C)

Ascorbic acid (AsA) was estimated as described by AOAC[15]. One gram fresh leaves homogenized with 10 ml of 0.4% oxalic acid in water and centrifuged (Remi 815) at 8000 rpm. Supernatant was used to test AsA content in samples. The 500 µl and 1000 µl aliquots of the supernatant in triplicate were maintained to 3 ml by 0.4% oxalic acid followed by the addition 7 ml of 2, 6-dichlorophenol indophenol dye (DCPIP) dye solution. The test mixture was properly mixed and its absorbance was recorded immediately at λmax 518 nm. Ascorbic acid was used as a standard in the range of 0 to 100 µg/ml. The amount of AsA was calculated by comparison with standard curve drawn under identical experimental conditions.

Determination of carotenoids

Total carotenoids were determined by the method of Jensen[16]. One gram sample was extracted with 100 ml of 80% methanol solution and centrifuged (Remi 815) at 4000 rpm for 30 min. The supernatant was concentrated to dryness. The residue was dissolved in 15 ml of diethyl ether and after addition of 15 ml of 10% methanolic KOH the mixture was washed with 5% ice-cold saline water to remove alkali. The free ether extract was dried over anhydrous sodium sulphate for 2 h. The ether extracts were filtered and its absorbance was measured at 450 nm with Spectrophotometer (Systronic, visiscan) by using ether as blank.

Determination of total phenol

Total phenolic contents in different extracts were measured by the method of Ragazzi and Veronese[17]. 10 mg plant extract was dissolved in 10 ml of 50% MeOH: H_2O (1:1, v/v), overnight at room temperature, 1.0 ml of Folin's reagent (1N) and 2.0 ml of Na_2CO_3 (20%) were added subsequently. The test mixture was mixed properly on cyclomixer, left at room temperature for 30 min and maintained to 25 ml with water. The absorbance of test mixture was measured at λ_{max} 725 nm.

RESULTS

Complex antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species. These include both enzymatic and non enzymatic antioxidants. The enzymatic antioxidants in the medicinal plants are represented in Table 2. The highest specific activity of ascorbate peroxidase was measured in *Cymbopogon citrates* (2.566 ± 0.254 µmol/min/mg/protein) and the lowest in *Cymbopogon martini* (0.526±0.0961). *Acorus calamus* (2.206±0.508 µmol/min/mg/protein) had the significantly higher specific activity of ascorbate peroxidase followed by *Cymbopogon nardus* (2.003±0.006) and *Pelargonium graveolens* (1.583±0.151). The specific activity of catalase perceived in studied medicinal plants was recorded maximum in *Pelargonium graveolens* (1195.69±0.012µmol/min/mg/protein) as compared to other plants while *Cymbopogon nardus* showed the least activity (622.00±0.003) of catalase. *Acorus calamus* displayed the little bit higher specific activity than the *Cymbopogon martini*. *Cymbopogon citrates* showed the less specific activity of catalase as compared to *Pelargonium graveolens* but expressed higher activity as compared to other three medicinal plants (Table 2). Superoxide dismutase (SOD) is the enzyme that catalyzes the more toxic superoxide anion radical to less toxic hydrogen peroxide. *Acorus calamus* revealed the highest

specific activity (3.59±0.22 µmol/min/mg/protein) of SOD where *Cymbopogon citrates* (0.172±0.022) generated the least. *Cymbopogon martini*s (2.276±0.013 µmol/min/mg/protein) possessed the bit higher specific activity of SOD than *Cymbopogon nardus* (2.173±0.732) but much more than *Pelargonium graveolens* (1.908±0.538) (Table 2).

Table 2
Specific activity of antioxidative enzymes in leaves of medicinal plants.

Plants Name	Ascorbate Peroxidase (µmol/min/mg/protein)	Catalase (µmol/min/mg/protein)	Superoxide dismutase (µ mol/min/mg/protein)
Acorus calamus	2.206±0.508	686.56±0.006	3.59±0.22
Pelargonium graveolens	1.583±0.151	1195.69±0.012	1.908±0.538
Cymbopogon martini	0.526±0.0961	671.76±0.0085	2.276±0.013
Cymbopogon nardus	2.003±0.006	622.00±0.003	2.173±0.732
Cymbopogon citratus	2.566±0.254	984.36±0.0165	0.172±0.022
S.E.	1.212	1.119	0.476
C.D.	1.345	2.351	0.999

Ascorbic acid is the strongest water soluble antioxidant. In the present study, *Cymbopogon martini* exhibited the highest concentration of ascorbic acid (592.09±0.0961 mg/100g) and *Cymbopogon nardus* (33.34±0.006) the lowest. *Acorus calamus* (407.42±0.508 mg/100g) had little higher concentration of ascorbic acid than *Cymbopogon citrates* (370.77±0.254) whereas *Pelargonium graveolens* (185.56±0.151 mg/100g) was observed to be the second lowest in ascorbic acid content (Table 3). *Acorus calamus* plant was found to possess the maximum (174.8± 3.930 mg/100g) carotenoids which was bit higher concentration than the *Cymbopogon martini* (170.37±0.540) whereas *Pelargonium graveolens* (15.57±0.498 mg/100g) contained the lowest concentration of carotenoids. Though, *Cymbopogon citrates* (93.06±3.577 mg/100g) which showed nearly half of the carotenoids than the *Acorus calamus*, comprised comparatively higher concentration than *Cymbopogon nardus* (76.08±2.857) and *Pelargonium graveolens* (15.57±0.498). The highest content of total phenol was recorded in *Pelargonium graveolens* (22.27±0.284 mg GAE ml^{-1}) plants where minimum in *Acorus calamus* (7.63±0.046). *Cymbopogon martini* was found average source of total phenol (10.01±0.098 mg GAE ml^{-1}) and observed bit higher than the *Cymbopogon nardus* (9.1±0.095). *Cymbopogon citrates* was seen to be the second highest in term of total phenol content (15.73±0.112 mg GAE ml^{-1}) which had one and half times higher content than the *Cymbopogon martini* and the 1.5 times lower than the *Pelargonium graveolens* (Table 3). Table 4 reveals the variable amount of protein and chlorophyll content in fresh leaves of studied medicinal plants. The highest content of protein was observed in *Pelargonium graveolens* (470±0.002µg/g) which showed much higher content than the *A. calamus, C. nardus* and *C. martini* plant leaves. *Cymbopogon citratus* (390±0.001µg/g) also displayed the appreciable amount of protein and exhibited higher than the *Acorus calamus*, *Cymbopogon martini* and *Cymbopogon nardus* that exhibited protein content in the range of 250-280µg/g (Table 4). The total chlorophyll

content was measured maximum in *Cymbopogon martini* (7.156±0.0148 mg/g) plant leaves while least was noted in *Acorus calamus* (3.216±0.0183). However, *Cymbopogon nardus* (6.547±0.0103 mg/g) contained higher chlorophyll as compared to *Cymbopogon citrates* (5.758±0.0155), *Pelargonium graveolens* (4.592±0.0114) and *Acorus calamus* (3.216±0.0183) (Table 4).

Table 3
Activity of non-enzymatic antioxidants in leaves of medicinal plants.

Plants Name	Ascorbic acid (mg/100g)	Total Phenol (mg GAE ml^{-1})	Total Carotenoids (mg/100g)
Acorus calamus	407.42±0.508	7.63±0.046	174.8±3.930
Pelargonium graveolens	185.56±0.151	22.27±0.284	15.57±0.498
Cymbopogon martini	592.09±0.0961	10.01±0.098	170.37±0.540
Cymbopogon nardus	33.34±0.006	9.1±0.095	76.08±2.857
Cymbopogon citratus	370.77±0.254	15.73±0.112	93.06±3.577
S.E.	1.445	1.923	2.084
C.D.	3.035	4.040	5.892

Table 4
Protein and Total Chlorophyll content in fresh leaves of Medicinal plants.

Plants Name	Protein (µg/g)	Total Chlorophyll (mg/g)
Acorus calamus	280±0.003	3.216±0.0183
Pelargonium graveolens	470±0.002	4.592±0.0114
Cymbopogon martini	270±1.00	7.156±0.0148
Cymbopogon nardus	250±0.003	6.547±0.0103
Cymbopogon citratus	390±0.001	5.758±0.0155
S.E.	0.002	2.789
C.D.	0.003	3.356

DISCUSSIONS

Reactive oxygen species (ROS) are generated due to many factors such as drought, cold, heat, herbicides and heavy metals, all of these factors lead to increase and accumulate ROS in plant cells[18]. Scientific research shows that ROS are harmful to the cell because they can raise the oxidative level through loss of cellular structure and

function[19]. ROS detoxification agents in cells include antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and polyphenol oxidase (PPO). Antioxidant systems in plants prevent or alleviate membrane peroxidation caused by ROS under high temperatures or other stressful conditions[20]. SOD and CAT constitute the suite of enzymatic defenses against oxidative stress[21]. SOD is located in chloroplasts, mitochondria, cytoplasm and peroxisomes, and acts as the first line of defense against ROS by dismutating O_2^- to H_2O_2[22]. Ascorbate peroxidase (APX) utilizes ascorbic acid as an electron donor in the neutralization of H_2O_2 both in cytosol and molecular compartments[18]. The studied plants had also developed the appreciable quantity of APX activity against environmental stress to protect the cellular damage. Selected plants evolved variable extent of SOD activity, which is the most indispensable enzyme for protecting the cells from the toxicity of the reactive oxygen species that are generated during aerobic respiration for energy production; it converts more toxic superoxide anion radicals to less toxic hydrogen peroxide[23]. One of the mechanisms *in vivo* is improving the endogenous cellular antioxidants mechanisms, such as up-regulation of the activity of superoxide dismutase[24]. *Acorus calamus* and *Cymbopogon martini* have the effective specific activity of SOD. Catalase is a tetrahedrical protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen[25]. In the studied medicinal plants, catalase activity was perceived in all plants where *Pelargonium graveolens* was effectively protected by oxidation of biotic and abiotic stress.

Natural ascorbic acid is vital for the body performance[26]. Lack of ascorbic acid impairs the normal formation of intercellular substances throughout the body, including collagen, bone matrix and tooth dentine Therefore, the clinical manifestations of scurvy hemorrhage from mucous membrane of the mouth and gastrointestinal tract, anemia, and pains in the joints can be related to the association of ascorbic acid and normal connective tissue metabolism[27]. This function of ascorbic acid also accounts for its requirement for normal wound healing. As a result of the availability of ascorbic acid in *Cymbopogon martini*, *Acorus calamus* and *Cymbopogon citratus* these plants are used in herbal medicine for the treatment of many diseases[28]. It is proved that carotenoids have a positive role on the epithelisation process and influence the cell cycle progression of the fibroblasts[29]. Carotenoids act as photoprotective agents and may reduce the risk of sunburns, photo-allergy and even some types of skin cancer[30]. The examined results shows *Acorus calamus* and *Cymbopogon martini* leaf is strong source of carotenoids and it can be a promising for use in pharmacological products designed for antioxidant activity. Phenolic compounds are an important group of secondary metabolites, which are synthesized by plants due to plant adaptation in response to biotic and abiotic stresses (infection, water stress, cold stress, high visible light)[31]. However, the antioxidant capacity of the plant extracts is mainly dependent on phenolic compounds. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts[32]. The phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties[33]. According to present study, the high phenolic content of *Pelargonium graveolens* can explain its high free radical scavenging activity. The chlorophyll incorporating into our diets will aid in growth and repair of tissues and also possess anti mutagenic and anti carcinogenic properties to a certain extent as it is a very good chelating agent due to its physical and chemical structure. Thus human don't have to rely on external chemicals or supplements for these benefits in keeping the body healthy and for increasing immunity[34]. Protein has excellent

potential as antioxidant additive in food because they can inhibit lipid peroxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radical and chelation of pro oxidative transition metals[35]. A variety of reactive oxygen species react readily with methionine residues in proteins to scavenge the reactive species. Thus, methionine residues may act as catalytic antioxidant, protecting both protein and other macromolecules[36].

CONCLUSION

This study supports the contention that traditional medicines remain a valuable source in the potential discovery of natural plant product pharmaceuticals. Significant free radical scavenging activity displayed by *A. calamus, P. graveolens, C. martini*, *C. nardus* and *C. citratus* provide a scientific validation for the traditional use of these plants. Studied medicinal plants are good sources of natural antioxidants and might be useful in treating the diseases associated with oxidative stress. Further work on isolation and identification of active compounds and its efficacy needs to be done.

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