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EVALUATION OF ANTIOXIDANT EFFECT OF VARIOUS EXTRACTS OF *LEUCAS ASPERA IN VITRO*

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ABSTRACT

Lamiaceae herbs known for their culinary properties are a rich source of potentially health-beneficial antioxidant polyphenols. The objective of this investigation was to evaluate and prove the *in vitro* antioxidant activity of five different extracts of aerial parts of the plant *Leucas aspera,* belonging to the family Lamiaceae on different models namely the DPPH free radical scavenging assay, Fe^{2+}- ascorbate induced lipid peroxidation, Nitric oxide scavenging activity, Total antioxidant equivalence and Hydroxyl radical scavenging activity by standardized methods. The results showed the ethanolic and hydroalcoholic extracts of *Leucas aspera* (EELA and HAELA) to exhibit maximum inhibition of free radical formation followed by EAELA, the ethyl acetate extract. The least antioxidant potential was exhibited by HELA and CELA, the n-hexane and chloroform extracts of *Leucas aspera*. The potential antioxidant activities *in vitro* may indicate their therapeutic and related beneficial properties in oxidative stress, a condition underlying many disorders.

KEYWORDS: ***Leucas aspera*, Free radicals, Lamiaceae, Antioxidants**

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INTRODUCTION

Leucas (Lamiaceae) is a large genus of herbs or undershrubs distributed throughout the tropical regions of the whole world. The genus *Leucas* comprises of about 80 species.[1] In India, 43 species are available.[2] Plants of genus *Leucas* are generally shrubs, or perennial herbs with woody root and/or stem-base. They have been widely employed by the traditional healers in various parts of Asia and Africa to cure many diseased conditions which insinuated that this genus might have immense potential for the discovery of new drugs or lead molecules. Some of the Indian species which have been proved for their medicinal values are *L. abyssicica, L. alluaudii, L. aspera, L. biflora, L. calostachys, L. cephalotes, L. hirta, L. indica, L. jamesii L. linifolia, L. lanata, L. lavendulaefolia, L. martini cenesis, L. mollissima, L. plukenetii, L. stelligera, L. urticaefolia* and *L. zeylanica*. Most of these plants serve as flavouring agents. Oxygen is an indispensable element for life. Oxidative properties of oxygen play a crucial role in various biological phenomena. It is paradoxical that oxygen even though essential for life can under certain situations have severe toxic effects on the system. Most of the potentially harmful effects of oxygen are due to the formation of free radicals from molecular oxygen.[3] Free radicals are atoms with an unpaired electron formed from molecules via the breakage of a chemical bond so that each fragment retains one electron. In biological systems, oxygen gives rise to a large number of free radicals collectively known as 'reactive oxygen species' (ROS) inclusive of the superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (HO^-), peroxyl (ROO^-), alkoxy (RO), and nitric oxide (NO).[4] Another group of reactive species are termed as 'reactive nitrogen species' (RNS) which includes molecules derived from nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$) produced via the enzymatic activity of inducible nitric oxide synthase (NOS^2) and NADPH oxidase respectively. ROS/RNS together cause oxidative damage of amino acids, lipids, proteins and DNA causing oxidative stress (OS). In a normal healthy human, the generation of ROS and RNS are effectively kept in check by the various levels of antioxidant defense which are capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being. Cellular damage induced by OS has been implicated in the etiology of a large number (>100) of human diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, neurodegenerative diseases and inflammatory conditions like rheumatoid arthritis as well as in the process of ageing. Antioxidants, exogenous or endogenous, synthetic or natural, prevent free radical formation by scavenging them or promoting their decomposition. Epidemiological and *in vitro* studies on medicinal plants strongly support the idea that plant constituents with antioxidant potential exert protective effects against OS in biological systems. There has been growing interest in natural antioxidants because they have greater application as nutraceuticals and phytoceuticals and a significant impact on the status of human health and disease prevention.[5] *Lamiaceae* herbs known for their culinary properties are a rich source of potentially health-beneficial antioxidant polyphenols. Their potential antioxidant action *in vitro* and in humans using a variety of parameters reflecting antioxidant status has been acknowledged.[6] There are still a large number of these plants and ayurvedic formulations whose antioxidant activities need to be examined in relation to their potential therapeutic and related beneficial properties. More recent assays also should be included to study the antioxidant properties of medicinal plants or their chemical constituents.[7] The objective of this investigation was to evaluate and prove the *in vitro* antioxidant activity of five different extracts of *Leucas aspera* on different models of *in vitro* studies.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of analytical grade. Solvents were obtained from Sisco Research Laboratories Pvt. Ltd., India. 1, 1-Diphenyl, 2-picryl hydrazyl (DPPH), gallic acid, catechin, tocopherol and curcumin were obtained from Sigma Chemicals, USA. The other chemicals inclusive of potassium ferricyanide, trichloroacetic acid, ferric chloride, ascorbic acid, potassium iodide, ammonium molybdate, sodium thiosulfate, Ethylenediamine tetraacetic acid (EDTA), ascorbic acid, 2-deoxy-2-ribose, trichloroacetic acid (TCA), mannitol, reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside (SNP), sulfanilamide, naphthyl ethylene diamine dihydrochloride (NED), sodium pyruvate were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Hydrogen peroxide, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$) were obtained from Merck, Mumbai, India. Ferrous sulfate was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India. Sodium nitrite was obtained from Qualigens Fine Chemicals, Mumbai, India.

Collection of Plant material

The aerial parts of the plant *L. aspera* were collected from local suburban areas of Kanchipuram district, Tamil Nadu, India during the months of January and February.The plant was taxonomically identified by Dr. N. Jayaraman, Director, National Institute of Herbal Science, Plant Anatomy Research Center, Tambaram, Chennai. Voucher specimen (PARC/2007/362) has been deposited in our college herbarium for future reference.

Preparation of Extracts

Shade dried and coarsely powdered aerial parts of *L. aspera* (1 kg) was subjected to exhaustive cold maceration in solvents of increasing polarity (n-hexane, chloroform, ethyl acetate and ethanol) successively for 72 hours, 48 hours, and 24 hours respectively. Simultaneously, 1 kg of the dried powder was subjected to exhaustive cold maceration in 50% ethanol for the same time span. The solvents were filtered, distilled under vacuum and dried in a vacuum dessicator to obtain the following extracts,

(i) n-hexane extract of *L. aspera* - HELA (yield-1.71%),
(ii) Chloroform extract of *L. aspera* – CELA (yield-3.13%)
(iii) Ethyl acetate extract of *L. aspera* - EAELA (yield-1.93%),
(iv) Ethanolic extract of *L. aspera* - EELA (yield-4.57%) and
(v) Hydroalcoholic extract of *L. aspera* - HAELA (yield-9.5%)

The extracts were prepared in varying concentrations using DMSO as solvent for the individual studies.

DPPH radical scavenging activity

The ability of the extracts to scavenge DPPH radicals was based on the method of Sreejayan and Rao, 1996.[8] To 1 ml of various concentrations of extract, 1ml methanolic solution of DPPH (0.1 mM) was added. An equal amount of methanol and DPPH served as control. After 20 minutes of incubation in the dark, absorbance was recorded at 517 nm. Standards treated similarly included curcumin. The experiment was performed in triplicates and percent inhibition was calculated.

Percent Inhibition= Control OD – Test OD/ Control OD x 100

Inhibition of Fe^{2+}- ascorbate induced Lipid peroxidation

The ability of the extracts to inhibit LPO *in vitro* was based on the method of Ohkawa et al., 1979.[9] Liver homogenate was prepared after the liver was quickly excised and washed several times with ice cold saline solution (with 0.15M KCl, pH 7.4). It was then homogenised in saline to prepare a 10% homogenate. Lipid peroxidation was initiated by the addition of 25 mM $FeSO_4$, 100 mM ascorbate and 10 mM KH_2 PO_4, the homogenates were incubated at 37°C for one hour with different concentrations of the extracts. 1 ml of 15% TCA and 0.375% TBA were added to all the tubes, placed in a boiling water bath for 30 minutes, centrifuged and the OD of the supernatant was measured at 532 nm.

Percent Inhibition= Control OD – Test OD/ Control OD x 100

Determination of reducing power

The reducing power of the extracts was determined according to the method of Oyaizu, 1986. [10] 1 ml of extract solution was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50º C for 20 minutes. 2.5 ml of 10% TCA was added and the tubes were centrifuged at 1500 g for 10 minutes. 1ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% freshly prepared ferric chloride solution. Absorbance was measured at 640 nm. The experiment was carried out in triplicates and average value taken for tabulation. Increased absorbance indicates increased reducing power given in terms of ascorbic acid equivalence.

Nitric oxide scavenging activity

The ability of the extracts to scavenge NO radicals was based on the method of Green et al., 1982.[11] Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH which interacts with oxygen to produce nitrate ions which is measured colorimetrically. 3 ml of reaction mixture containing 2 ml of sodium nitro prusside (10 mM) in PBS and 1 ml of various concentrations of extracts were incubated at 37º C for 4 hours. Control tubes do not contain sample and are treated similarly. After incubation, 0.5 ml of Griess' reagent was added. The absorbance of the chromophore was read at 546 nm. The percentage inhibition of nitric oxide generation was measured by comparing the absorbance value of control and those of samples. Curcumin was used as a standard. The experiment was carried out in triplicates and average value taken for tabulation. The percentage inhibition was calculated using the formula

% NO inhibition = Control OD – Test OD/ Control OD x 100

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extracts was determined by the method of Halliwel et al., 1985.[12] Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to the cell. The reaction mixture consisted of deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), and the extract (from 62.5-2000 µg/ml) in a final volume of 1 ml. After incubation for one hour at 37 ^oC, the deoxyribose degradation was measured as TBARS by the method of Ohkawa et al., 1979.[9]

% Inhibition of hydroxyl radical= Control OD – Test OD/ Control OD x 100

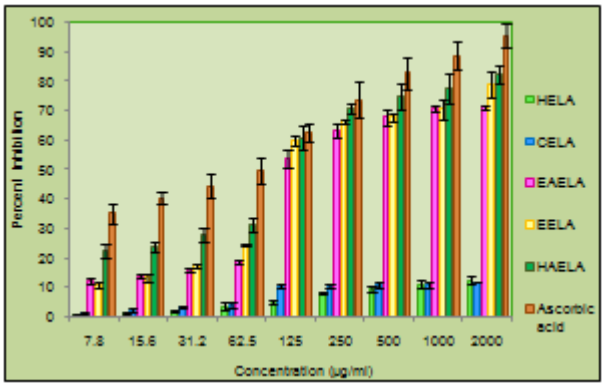
Total antioxidant equivalence

Total antioxidant activity of the extracts was determined by the method of Prieto et al., 1999.[13] 0.1 ml of the extract (10 mg/ml) dissolved in water was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 ºC for 90 minutes. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. Tocopherol was used as the standard and the total antioxidant activity is expressed in terms of gram equivalence of tocopherol.

RESULTS AND DISCUSSION

In nature, there exists a dynamic balance between the amount of free radicals produced in the body and antioxidants that scavenge or quench them to protect the body against deleterious effects. Antioxidants capable of stabilizing or deactivating free radicals under normal physiological conditions may be insufficient to neutralize free radicals generated during OS. Therefore, it is essential to enrich our diet with antioxidants to protect against harmful diseases. Hence there has been an increased interest in the food industry and in preventive medicine in the development of "natural antioxidants" from plant materials[14] thereby necessitating the evaluation of the antioxidant property of extracts of plants used in folk medicine. This is partly due to renewed interests in the use of concoctions and/or decoctions made from these plants in alternative medicine and to scientific evidences implicating positive roles of the antioxidant components of these plants being protective against ROS related to pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity. Many synthetic antioxidant compounds such as BHT, ascorbic acid have been demonstrated to exhibit toxic and /or mutagenic effects; hence attention is now being shifted towards the naturally occurring antioxidants derived from plants.[15] The free radical scavenging ability of various extracts of *L. aspera* was confirmed in the present investigation. Owing to the complexity of the antioxidant resources and their mechanism of actions, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample and a combination of different methods is necessary.

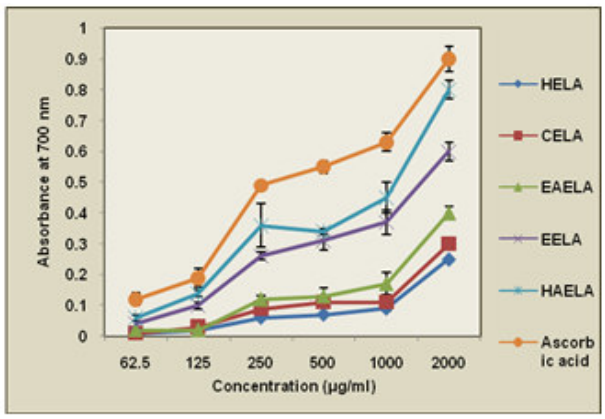
DPPH Radical scavenging activity of extracts of L. aspera



Graph 1
DPPH Radical scavenging activity of extracts of L. aspera

DPPH free radical scavenging assay can be helpful for primary screening and finding of novel antioxidants.[5] This is the most widely reported method for screening of antioxidant activity of many plant drugs and the first antioxidant method set up in laboratories. DPPH assay method is based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 516 nm, which is proportional to the concentration of free radical scavenger added to DPPH reagent solution. The results are highly dependent on the reaction time, the concentration of DPPH used and the antioxidant(s) in the assay mixture.[6] In the present study, the DPPH radical scavenging activities of various investigated extracts from aerial parts of *L. aspera* were in the order of HAELA (70.62%) > EELA (66.6%) > EAEALA (63.33%) > CELA (10.48%) > HELA (8.39%) at a concentration of 250 µg/ml while the standard antioxidant ascorbic acid exhibited 73.8% inhibition at the same concentration, as shown in Graph 1. These data suggested that HAELA, EELA and EAELA have remarkable ability to scavenge radicals in a dose dependant manner. This capacity of the plant in scavenging DPPH has been established in earlier studies.[16, 17]

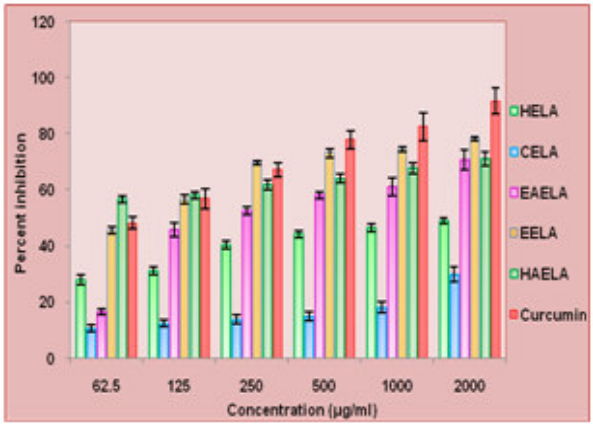
Reducing power of extracts of L. aspera



Graph 2
Reducing power of extracts of L. aspera

The reducing power method which is based on the principle of increase in the absorbance of the reaction mixture may serve as a significant indicator of potential antioxidant activity of the extracts. In this method, antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700 nm. Moreover, result of the assay is correlated with total phenolic content and ferric ion reducing power. Literature is scarce on the reducing power of *L. aspera.* As shown in graph 2, reducing power of all the extracts namely HELA, CELA, EAELA, EELA and HAELA displayed an increasing trend with increasing concentrations as indicated by the increase in absorbance of the reaction mixture. The absorbance was found to be 0.06, 0.14, 0.36, 0.34, 0.45 for the five extracts and 0.6 for ascorbic acid at a concentration of 1000 µg/ml respectively.

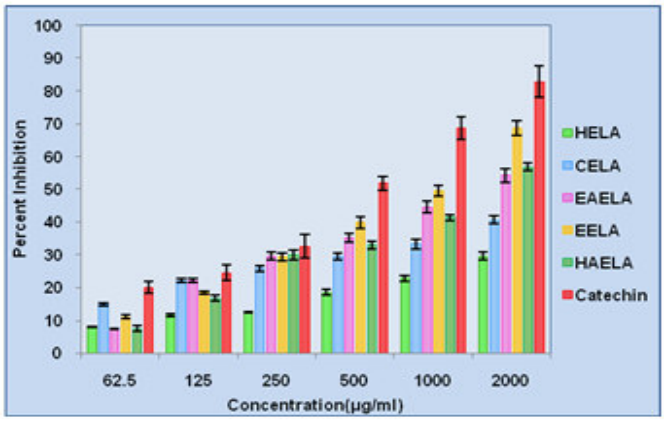
Inhibition of Fe^{2+}- ascorbate induced Lipid peroxidation



Graph 3
Inhibition of Fe^{2+}- ascorbate induced Lipid peroxidation

Lipid peroxidation has been defined as the oxidation of polyunsaturated fatty acids (PUFA) by a radical-induced chain reaction[18] to form a lipid hydroperoxide (ROOH) which then reacts further. Free oxygen radicals and lipid peroxides are shortlived products and difficult to measure directly. Malondialdehyde on the other hand is a more stable and long-lived degradative product of lipid peroxide and is often assayed as reflecting lipid peroxidation levels.[19] Consistent evidence is available for the reaction between MDA and cellular macromolecules such as proteins, RNA and DNA. MDA reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine which may be mutagenic.The results showed that ethanolic and hydroalcoholic extracts (EELA-74.4% and HAELA-67.78%) to exhibit maximum inhibition of lipid peroxidation followed by ethyl acetate extract (61.23%) and n-Hexane (46.58%) extracts at a concentration of 1000 µg/ml. The results were compared with that of standard curcumin, a potent antioxidant. The propagation of lipid peroxidation is broken by either enzymatic inactivation of ROS involved in chain reaction or non-enzymatic reactions due to the intervention of free radical scavenger and antioxidant compounds probably present in the investigated extracts.[20]

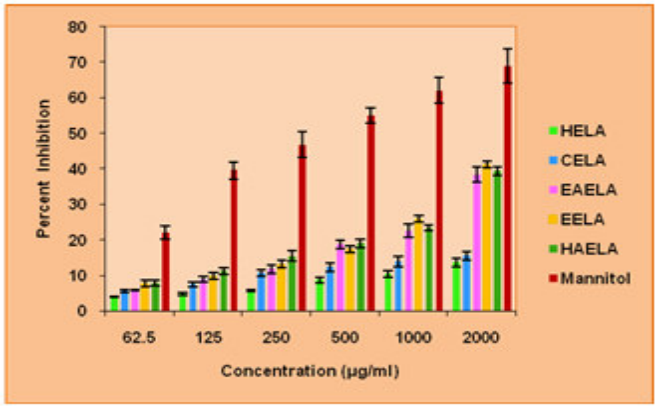
Nitric oxide scavenging activity of extracts of L.aspera



Graph 4
Nitric oxide scavenging activity of extracts of L.aspera

Nitric oxide (NO) identified initially as a vasodilator, has multiple functions and complex physiological and pathophysiological roles that have been revealed. In particular, nitric oxide (NO) and reactive nitrogen species (RNS), derived from the interaction of NO with oxygen or ROS, have both been reported to participate in the development of oxidative tissue/cellular damage, which has been established as a mechanism of tissue damage.[21,22] Hence *in vitro* inhibition of nitric oxide radical is also a measure of antioxidant activity. This method is based on the inhibition of nitric oxide radical generated from sodium nitroprusside in buffered saline and measured by Griess's reagent. The activity is expressed as % reduction of nitric oxide. Graph 4 illustrates the significant inhibition of nitric oxide radical generation by the extracts of *L. aspera* as well as standard catechin and their maximum inhibition at 2000µg/ml was found to be 29.7%, 40.74%, 54.2%, 68.74%, 56.8% for HELA, CELA, EAELA, EELA, HAELA and 82.8% for catechin standard respectively. The structural feature of flavonoids that might be responsible for scavenging NO has been examined in earlier studies. For instance, the catechol group is a basic requirement for excellent NO scavenging and gallic acid linked to flavan-3-ol is more important in the scavenging of NO by catechins- a group of flavonids.[21]

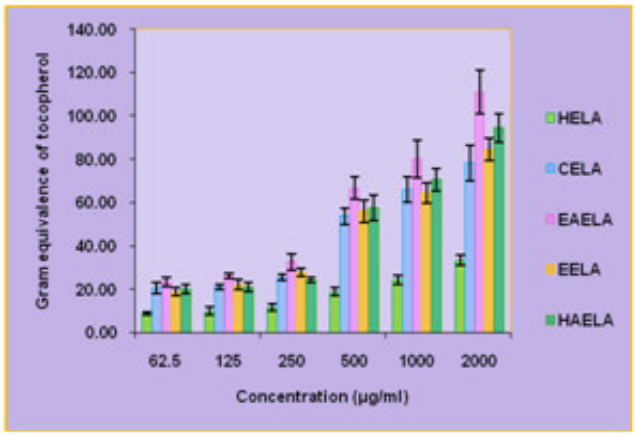
Hydroxyl radical scavenging activity of extracts of L. aspera



Graph 5
Hydroxyl radical scavenging activity of extracts of L. aspera

Hydroxyl radical is one of the potent ROS in biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cells.[12] Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity. The model used is ascorbic acid-iron-EDTA, in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals *in vitro* using the Fenton reaction. Highly reactive OH^- can cause oxidative damage to DNA, lipids and proteins.[23] This assay has been designed for testing the capability of antioxidants to scavenge OH^- radicals generated in reactions between hydrogen peroxide and iron ions. The approach of generating and testing a radical with real biological relevance is ideal.[6] The various extracts of *L. aspera* and mannitol, the standard compound showed differences in their ability to scavenge OH^- radicals (Graph 5). At a concentration of 1000 µg/ml, the extracts HELA, CELA, EAELA, EELA and HAELA showed 10.4%, 13.92%, 22.54%, 25.98%, 23.45% inhibition respectively and mannitol, 62.14% inhibition. The extracts were found to exhibit hydroxyl radical scavenging activity in a dose dependent manner.

Total antioxidant capacity of extracts of L.aspera



Graph 6
Total antioxidant capacity of extracts of L.aspera

Total antioxidant capacity is usually reported as ascorbic acid equivalents or gram equivalents of tocopherol. The lipophilic and hydrophilic components in plants contribute a good measure of total antioxidant capacity. The best health and nutrition can be achieved by consuming fruits and vegetables with high antioxidant capacities. The major types of phenolic compounds in the medicinal plants were hydrolysable tannins and phenolic acid (gallic acid) which serve as antioxidants with considerable health benefits. This method determines the antioxidant capacity of the extracts, through the formation of phospho molybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate-Mo (V) complex at acidic pH. The phosphomolybdenum method, in combination with hexane monophasic extraction, has also been adapted for the specific determination of vitamin E in seeds. Since the antioxidant activity was expressed as the number of equivalents of tocopherol, the study revealed that the antioxidant activity of the extracts increased with increasing concentration of the plant extracts. Maximum antioxidant activity was observed at a concentration of 2000µg/ml where EAELA, HAELA, EELA, CELA and HELA contained 111.11, 94.44, 84.44, 78.33 and 33.3 gram equivalents of tocopherol as shown in the Graph 6. Different antioxidant and radical scavenging activity may partly be due to wide variety of antioxidant constituents such as phenolics, flavonoids and ascorbate. In addition, two types of antioxidants namely, inhibitors of free radicals which initiate oxidation and inhibitors of free radical chain propagation reactions, are known. Direct anti-radical, chain-breaking of the free radical propagation, as well as interaction with transition metals can also play a role. Also, the inhibition of ROS-generating enzymes such as xanthine oxidase or inducible nitric oxide synthase is possible.[24] It was also revealed that the extracts obtained by polar organic solvents (hydroalcohol and ethanol) prove to be potentially active than those obtained by non-polar organic solvents (chloroform and n-hexane) as shown in earlier studies.[20] However, the chemical constituents present in the extract, which are responsible for this activity, need to be investigated. The phytochemical tests indicated the presence of alkaloids, glycosides, tannins, and flavonoids in the extracts. Several of such compounds are known to possess potent antioxidant activity. Some of these constituents have already been isolated from this plant.[25] Hence, the observed antioxidant activity may be due to the presence of any of these constituents.

CONCLUSION

The findings of this study support the view that *L. aspera* is a promising source of potential antioxidants and may be efficient in the prevention of diseases involving free radicals and oxidative stress. Further studies are warranted for the isolation and characterization of lead molecules with antioxidant properties from the plant.

CONFLICT OF INTEREST

Conflict of interest declared none.

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