Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Free radical scavenging potential of *Lagenaria siceraria* (Molina) Standl fruits extract

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ARTICLE INFO

Article history:
Received 24 June 2012
Received in revised form 31 August 2012
Accepted 5 October 2012
Available online 20 January 2013

Keywords:
Lagenaria siceraria
Antioxidant
DPPH
ABTS
Free radical

ABSTRACT

Objective: To elucidate free radical scavenging activity of ethanolic extract *Lagenaria siceraria* (*L. siceraria*) (Molina) fruit. **Methods:** The free radical scavenging activity of the *L. siceraria* (Molina) fruit extract was assayed by using α, α-diphenyl-β-picrylhydrazyl (DPPH), 2,20-azinobis 3-ethyl benzothiazoline-6-sulfonate (ABTS), FRAP, reducing power, chelating ability and β-carotene bleaching assay. **Results:** The IC_{50} values of DPPH and ABTS radical-scavenging activity was found to be 1.95 mg/mL and 19 mg/mL, respectively. In ferrous chelation assay, the percentage of inhibition was found to be 89.21%. The reducing power of ethanolic extract of *L. siceraria* (Molina) fruit was 0.068 at 1 mg/mL and increased to 0.192 at 5 mg/mL. The β-carotene linoleate bleaching assay was 46.7% at 5 mg/mL and antioxidant activity using FRAP at 0.305 for 1 mg/mL to 0.969 for 5 mg/mL. **Conclusions:** The results indicate that *L. siceraria* (Molina) fruit could be an important sources of natural radical scavengers.

1. Introduction

Plants rich in antioxidants, including polyphenolic compounds, tocopherols, vitamin C and carotenoids, are attracting to the food industry as replacements for synthetic ones, which use is being restricted due to safety concerns. The synthetic antioxidants have been widely used to control lipid oxidative rancidity in foods, which is a major cause of quality deterioration, nutritional losses, off-flavour development and discoloration. Besides prolonging the shelf-life of food products, these compounds are able to retard the progress of many oxidative stress-related chronic diseases in man. Therefore, dietary antioxidants also have an important role as nutraceuticals due to their role in protecting the body from free radicals, reactive oxygen species and reactive nitrogen species, which are derived either from normal metabolic processes or from external sources[1]. This protection is likely to involve several mechanisms of action, including inhibition of the generation of free radicals, enhancement of the scavenging capacity against free radicals, reducing capacity and metal chelating ability. The antioxidant activity assays usually involve these reactions. Based on the antioxidant activity assays and the lipidic system used as substrate, a wide range of activities can be determined[2].

Natural antioxidants have a wide range of biochemical activities, including inhibition of reactive oxygen species (ROS) generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential. Vegetables and fruits are rich sources of antioxidants, such as vitamin A, vitamin C, vitamin E, carotenoids, polyphenolic compounds and flavonoids, which prevent free radical damage, reducing risk of chronic diseases. Thus, the consumption of dietary antioxidants from these sources is beneficial in preventing cardiovascular diseases, especially atherosclerosis[3].

Human body has multiple mechanisms especially

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Foundation project: It is supported by University Grants Commission (UGC – File No. 2678/09 (MRP/UGC – SERO), New Delhi, India.

enzymatic and non-enzymatic antioxidant systems to protect the cellular molecules against ROS induced damage. However, the innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS in human body. Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they possess potential health risks and toxic properties to human health and should be replaced with natural antioxidants. Thus a need for identifying alternative natural and safe sources of food antioxidants has been created, and the search for natural antioxidants, especially of plant origin, has notably increased in recent years. Hence, compounds especially from natural sources capable of protecting against ROS mediated damage may have potential application in prevention and/or curing of diseases[4].

Oxygen centered free radicals and other ROS can be generated as by-products during oxidative progresses of living organisms. Many human diseases, including accelerated ageing, cancer, cardiovascular disease, neurodegenerative disease and inflammation, are linked to excessive amounts of free radicals. The antioxidants are necessary to cure these diseases. How-ever, the synthetic antioxidants might be unsafe. Therefore, more attentions are drawn to searching for natural antioxidants from medicinal and dietary plants to prevent oxidative damage[5].

Furthermore, there are ample evidences that reactive oxygen/nitrogen species generated in the human body can cause oxidative damages associated with many degenerative diseases such as atherosclerosis, coronary heart diseases, aging and cancer. It has been recognized that there is an inverse association between consumption of some fruits and vegetables and morbidity and mortality from degenerative diseases, which could be partly attributed to their antioxidants[6]. The health promoting effect of antioxidants from plants is thought to arise from their potential effects on the reactive oxygen/nitrogen species. In addition, antioxidants have been widely used in food industry to prolong the shelf life.

Traditionally, medicinal plants are used for more than one disease and they may possess very high bioactivity against common targets. In this context, antioxidant property has significance as it can target ROS implicated in many disease conditions. Therefore, the present study has been carried out was to explore the antioxidant potential of ethanolic extract of *Lagenaria siceraria* (*L. siceraria*) (Mol) fruits (EELSF) and to make a comparison taking into account their ability to target multiple radical species such as DPPH, ABTS, chelating ability, reducing power, β-carotene bleaching and FRAP assay.

2. Materials and Methods

2.1. Collection and authentication of *L. siceraria* (Mol) fruit

The *L. siceraria* (Molina) fruit were collected in the month of august to December from the local market of Thiruchengodu, Tamil Nadu, India, and authenticated by the authority of the Botanical Survey of India (BSI), Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. A voucher specimen (specimen No. BSI/SC/5/23/07-08/Tech-1579) has been deposited in the same department for the further reference.

2.2. Preparation of plant extract

The fresh fruits of *L. siceraria* (Molina) were cut into pieces and dried in a shade to constant weight. The dried pieces were then ground into powder using an electrical blender. The 100 g of dried coarsely powdered fruits were extracted in 90% ethanol for 4 days at 50-60 ℃ in a soxhlet apparatus. The mixtures were then filtered. The filtrate was concentrated on a rotary evaporator at 45 ℃ for ethanol elimination and the extracts were kept in sterile bottles under refrigerated conditions until use.

2.3. Source of chemicals

The chemicals used in the present study were of analytical reagent grade. It was purchased from SD fine chem., Himedia and Qualigens, India.

2.4. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH free radical scavenging activity of the extracts was measured according to the method of Esa *et al*[7]. Stock solution of each mushroom extract (50 mg/mL) was diluted to a concentration in the range of 0.1 to 50 mg/mL. For the test, 3.9 mL of 0.06 mM DPPH radical (Sigma) was added to 0.1 mL of mushroom extract. Reaction mixture was vortexed and absorbance was measured at 515 nm using a spectrophotometer with methanol as the blank. The decrease in absorbance was monitored at 0 min, 1 min, 2 min, and every 15 min until the reaction has reached a plateau. The time taken to reach the steady state was determined by one-way analysis of variance (ANOVA). The DPPH free radical scavenging activity, expressed as percentage of radical scavenging activity, was calculated as follows: Radical scavenging activity (SA) = $A_0 - A_s / A_0 \times 100$, where A_0 is the absorbance of 0.06 mM methanolic DPPH only whereas A_s is the absorbance of the reaction mixture.

2.5. 2, 2′-Azinobis 3-ethylbenzothiazoline-6-sulphonate (ABTS) free radical scavenging activity

The determination of $ABTS^+$ radical scavenging was assessed as described by Anagnostopoulou *et al*[8]. The $ABTS^+$ radical was generated by reacting an (7 mmol/L) $ABTS^+$ aqueous solution with $K_2S_2O_8$ (2.45 mmol/L, final concentration) in the dark for 12-16 h and adjusting the Abs 734 nm to 0.700 at ambient temperature. Extract was diluted such that a 15 mL sample, when added to 1.485 mL $ABTS^+$

solution, resulted in a 20%-80% inhibition of the absorbance. Later 15 mL extract/Trolox/standards were added to 1.485 mL ABTS+, the absorbance at 734 nm was recorded at 1 min after initial mixing and subsequently at 1 min intervals (30 min). The percentage inhibition was plotted as a function of concentration and the Trolox equivalent antioxidant capacity (TEAC) was calculated against a Trolox calibration curve.

2.6. Chelating power

The ability of the extract to chelate iron (Ⅱ) was estimated according to the method of Dastmalchi *et al*[9]. An aliquot of each sample (200 μL) was mixed with 100 μL of $FeCl_2$• $2H_2O$ (2.0 mmol/L) and 900 μL of MeOH. After 5 min incubation, the reaction was initiated by the addition of 400 mLof ferrozione (5.0 mmol/L). After 10 min incubation, the absorbance at 562 nm was recorded. The chelating activity (%) was calculated as the following equation:

Chelating activity (%) = [Abs control − (Abs sample /Abs control)] × 100. EC_{50} value is the effective concentration that could chelate 50% of iron (Ⅱ). Ascorbic acid was used as controls.

2.7. Reducing power activity

Various concentrations of extracts (2.5 mL) were mixed with sodium phosphate buffer (pH 6.6, 200 mM, 2.5 mL) and potassium ferricyanide (1% w/v, 2.5 mL). The mixture was incubated at 50 ℃ for 20 min. Trichloroacetic acid (10%, 2.5 mL) was added, and the mixture was centrifuged at 1 000 rpm for 8 min. The upper layer (5 mL) was mixed with deionised water (5 mL) and ferric chloride (0.1%, 1 mL), and the absorbance was measured spectrophotometrically at 700 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at k = 700 nm against extract concentration. BHA and α-tocopherol were used as standards[10].

2.8. β-Carotene bleaching (BCB) assay

A modified method of the BCB assay described by Lim and Quah[11] was employed in this study. Three millilitre of β-carotene (5 mg/50 mL in chloroform) was added to linoleic acid (40 mg) and Tween 40 (400 mg). The chloroform in the mixture was evaporated, followed by addition of oxygenated ultra-pure water (100 mL) to prepare the β -carotene/linoleic acid emulsion. The emulsion was mixed well and the initial absorbance of the emulsion was measured at 470 and 700 nm. Aliquots of the emulsion (3 mL) were added to the extracts (10- 100 μL), and were then incubated at 50 ℃ for 60 min. The absorbance measurements were taken and antioxidant activity was calculated according to the formula reported by Lim and Quah. In this assay, quercetin was used as a positive control.

2.9. Ferric-reducing antioxidant power (FRAP)

The FRAP of each standard solution was measured according to a modified protocol developed by Benzie and Strain[12]. To prepare the FRAP reagent, a mixture of 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM ferric chloride (10:1:1, v/v/v) was made. To 1.9 mL of reagent was added 0.1 mL of extract. The absorption maximum (593 nm) were taken using a Shimadzu UV visible 2501 spectrophotometer, quercetin were used as a positive control. Reagent blank reading, using 0.175 mL of FRAP reagent ($A_{reagent\ blank}$) and sample blank reading, using sample and acetate buffer ($A_{sample\ blank}$) were taken. The change in absorbance was calculated.

3. Results

There is increasing interest in natural antioxidant products for use as medicines and food additives[13]. The phytochemicals from fruits and vegetables hold promising potential in the development of health foods, nutritional supplements and herbal medicines for the application as antioxidants[14]. Vegetables and fruits are considered to be good sources of functional ingredients. Many studies have shown that antioxidants, present in plants at high levels, are the compounds responsible for these functionalities. Antioxidants or molecules with radical scavenging capacity are thought to exert a potential protective effect against free radical damage. These biomolecules contribute to prevention of coronary and vascular diseases and of tumor formation by inhibiting oxidative reactions. Due to the presence of different antioxidant components in the crude extracts of biological tissue samples, it is relatively difficult several assay methods have been developed and applied in recent years to screen and evaluate the total antioxidant activity of such samples[15]. In the present work, the DPPH, ABTS, Ferrous ion chelation, Reducing Power, β-carotene bleaching and FRAP radical-scavenging assay were successfully used for the evaluation of antioxidant activity of the crude extract, derived from *L. siceraria* (Molina).

The assays were performed in the whole extract, since it could be more beneficial than isolated constituents; a bioactive individual component can change its properties in the presence of other compounds present in the extracts. According to Liu[16], additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent bioactive properties and the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods. This explains why no single antioxidant can replace the combination of natural phytochemicals to achieve health benefits.

3.1. DPPH radical scavenging assay

Figure 1 shows the radical scavenging activity on DPPH radicals assay is extensively used as a basic screening

method for testing the antiradical activity of fruit extract. The scavenging activity of EELSF on DPPH radicals increased with increasing concentrations (1-5 mg). IC_{50} value (the amount of antioxidant material required to scavenge 50% of free radical in the assay system) of EELSF was observed as 1.95 mg/mL.

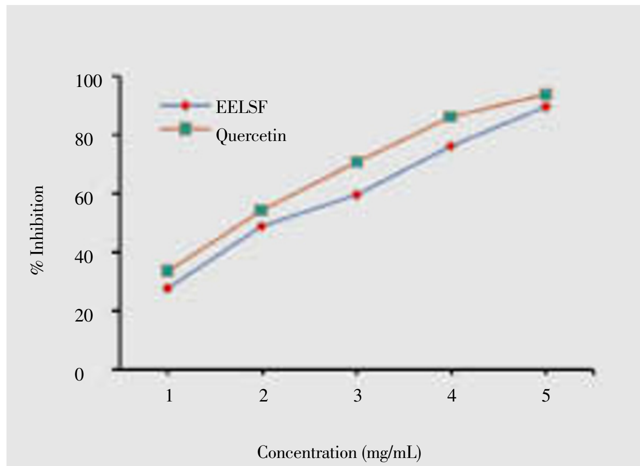

Figure 1. DPPH scavenging activity of *L. siceraria* (Molina) fruit extract.

3.2. ABTS radical scavenging assay

Figure 2 depicts the ABTS radical scavenging activities of EELSF, which shows antioxidant activities proving their capacity to scavenge ABTS radical cation. The ABTS radical scavenging property of EELSF increased from 33.23% at 10 mg/mL to 95.98% at 50 mg/mL concentration. The extract showed better activity in quenching ABTS with an IC_{50} value of 19 mg/mL.

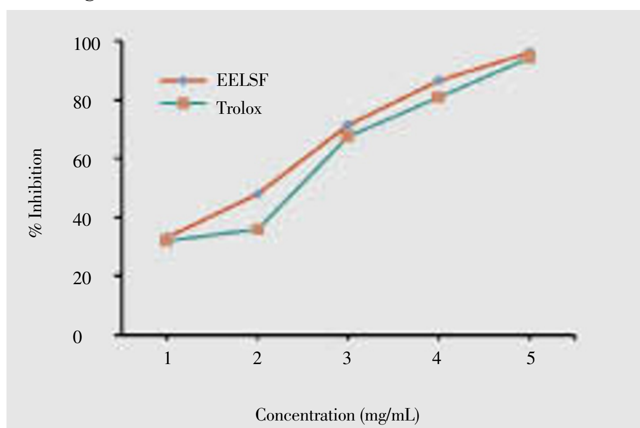

Figure 2. ABTS scavenging activity of *L. siceraria* (Molina) fruit extract.

3.3. Chelating effect on ferrous ion

The metal chelating activities of EELSF were concentration dependent. The absorbance of Fe^{2+}-ferrozine complex was linearly decreased with concentration dependently from 20 mg/mL. The percentages of metal scavenging capacity at the concentration of 100 mg/mL of EELSF were found to be 89.21% (Figure 3).

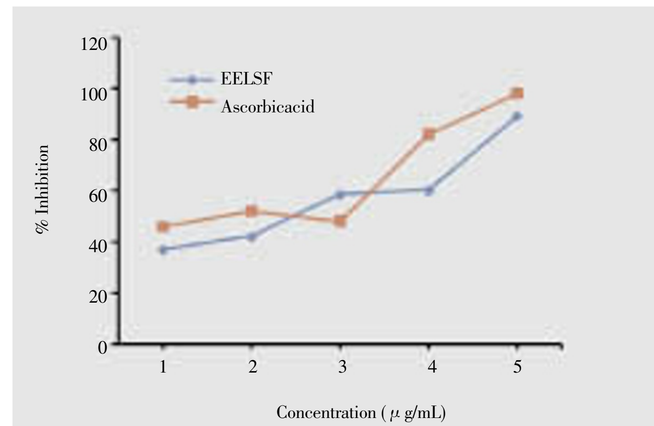

Figure 3. Chelating ability *L. siceraria* (Molina) fruit extract.

3.4. Reducing power activity

The results show that the fruits of *L. siceraria* (Molina) possess antioxidant properties which could react with free radicals to stabilize and terminate radical chain reactions. High values of reducing power at low concentrations of the extract, at lg/ml level, were obtained. The reducing power of ethanolic extract of *L. siceraria* (Molina) fruit was 0.068 at 1 mg/mL and increased to 0.192 at 5 mg/mL (Figure 4).

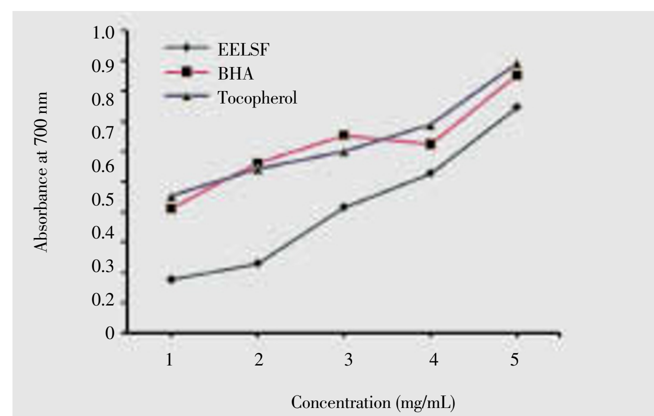

Figure 4. Reducing power of *L. siceraria* (Mol) fruit extract.

3.5. β -carotene bleaching assay and FRAP

Figure 5 shows the antioxidant activity of the EELSF as measured by the bleaching of β -carotene. Their antioxidant activities were 46.7% at 5 mg/mL. The protection of β -carotene bleaching provided by TBHQ standard reached 82.2% at 2 mg/mL and was slightly more efficient than the samples. It is probable that the antioxidative components in the fruit extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. Figure 6 shows the antioxidant activity of the EELSF as measured by FRAP activity. Their antioxidant activity is evident that there

is an increase in absorbance with increasing concentration. The absorbance increased from 0.305 for 1 mg/mL to 0.969 for 5 mg/mL.

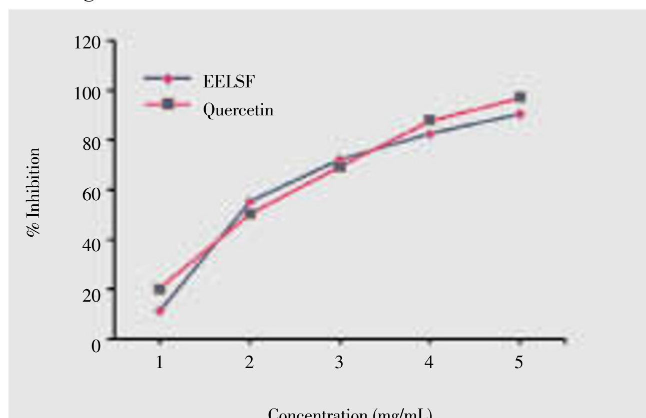

Figure 5. β – carotene bleaching ability of *L. siceraria* (Mol) fruit extract.

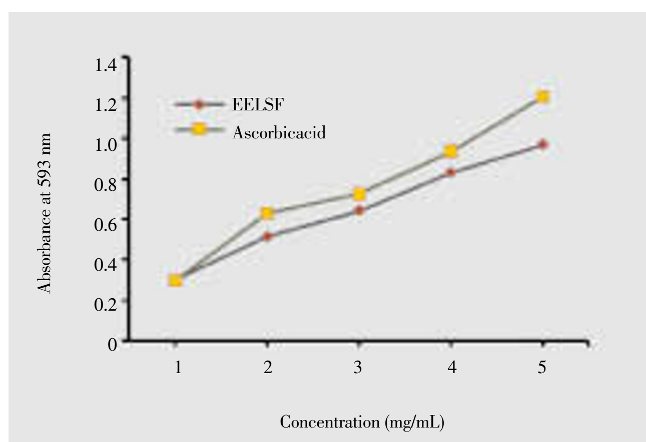

Figure 6. FRAP of *L. siceraria* (Mol) fruit extract.

4. Discussion

DPPH is a stable free radical that possesses a characteristic absorption maximum at 517 nm, which is diminished in the presence of a compound (*i.e.* antioxidants) capable of reducing it to its hydrazine form by hydrogen/electron donation. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation[17]. In this assay, results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wavelength. EELSF exhibited strong free radical scavenging activity on DPPH assay.

DPPH is a stable free radical that accepts an electron of hydrogen radical to become a stable diamagnetic molecule. The reduction in DPPH radical was determined by the decrease in its absorbance at 517 nm induced by antioxidants. From the result obtained it may be postulated that EELSF reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles. DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution losses colour stoichometrically depending on the number of electrons taken up[18].

It was found that the DPPH radical scavenging activity was reduced by the hydrogen donating ability. The results revealed that the DPPH radical scavenging activity might be attributed to the electron donating ability. The DPPH assay method is based on the reduction of DPPH, a stable free radical. With the odd electron, the free radical DPPH gives a maximum absorption at 517 nm by visible spectroscopy (purple colour). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, *e.g.*, a free radical-scavenging antioxidant, the absorption strength is decreased, and the resulting decolorization (yellow colour) is stoichiometric with respect to the number of electrons captured[19]. This reaction has been widely used to investigate the ability of plant extracts and fractions and/or pure compounds of those, to act as free radical-scavengers or hydrogen donors.

The ABTS assay is based on the inhibition by antioxidants of the absorbance of the radical cation of ABTS, which has a characteristic long-wavelength absorption spectrum showing maxima at 660, 734 and 820 nm. Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of various substances. The experiments are carried out using a decolorization assay, which involves the generation of the ABTS chromophore by the oxidation of ABTS with potassium persulphate. Trolox, a water-soluble analogue of vitamin E is used as the reference standard. The assay has been widely used in many recent studies related to detection of antioxidant property of plant.

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals[20]. Higher concentrations of the extracts were more effective in quenching free radicals in the system. The results obtained clearly imply that the ethanol extracts of plant species inhibit the radical or scavenge the radical in a concentration dependent manner.

One of the mechanisms of antioxidative action is chelation of transition metals, thus preventing catalysis of hydroperoxide decomposition and Fenton-type reactions. It has been well established that chelating agents stabilize transition metals and reduce their availability as catalysts, to inhibit the production of the first few free radicals and consequently suppress lipid peroxidation in biological and food systems. The fractions and standard antioxidant compounds interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine[21].

The ethanol extract of *L. siceraria* (Molina) fruits demonstrated a strong chelating activity on ferrous ions with

increased concentration. Ferrozione can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents such as antioxidant compounds, the complex formation is disrupted resulting in a decrease in the red colour of the complex, measurement of colour reduction therefore allows an estimation of the metal chelating activity of the coexisting chelator. In this assay, EELSF interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine.

Metal chelating capacity was significant, since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce redox potential thereby stabilizing the oxidized form of the metal ion. The data obtained reveal that EELSF demonstrate a marked capacity for iron binding, suggesting that their action as a peroxidation protector may be related to its iron binding capacity.

Reducing power is widely used to evaluate the antioxidant activity of polyphenols. The reducing power is generally associated with the presence of reductones, which exerts antioxidant action by breaking the free radical chain by donating a hydrogen atom[22]. The reducing power of a compound is related to its electron transfer ability and may, therefore, serve as a significant indicator of its antioxidant activity[23].

In the reducing power assay, the extracts prepared by EELSF displayed a concentration-dependent antioxidant potential. In this assay, the presence of reducers (antioxidants) in the extracts causes the reduction of the Fe^{3+}/ ferricyanide complex to the ferrous form, leading to a color change of the test solution from yellow to various shades of green and blue, depending to the reducing power capacity of each tested extracts. Therefore, Fe^{2+} concentration can be monitorized by measuring the formation of Perl's Prussian blue at 700 nm. Increased absorbance at 700 nm indicates an increase in reducing power. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts including tea[24].

The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radicals from linoleic acid. The linoleic acid free radical attacks the highly unsaturated β -carotene models. The presence of different antioxidants can hinder the extent of β-carotene-bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system. Accordingly, the absorbance decreased rapidly in samples without antioxidant whereas, in the presence of an antioxidant, they retained their colour, and thus absorbance, for a longer time. Antioxidant activities of the fruit extracts increased with their increasing concentration.

In this assay, oxidation of linoleic acid produces hydroperoxide-derived free radicals which attack the chromophore of β -carotene, resulting in a bleaching of the reaction emulsion. An extract capable of retarding/ inhibiting the oxidation of β -carotene may be described as a free radical scavenger and primary antioxidant[25]. According to the β -carotene-linoleic acid bleaching data, the extracts are capable of scavenging free radicals in a complex heterogenous medium. This suggests that the extracts may have potential use as antioxidative preservatives in emulsion-type systems. β-carotene shows strong biological activity and is physiologically an important compound, if β -carotene is decomposed before its intake, its biological functions in the body would not be observed. However, its 11 pairs of double bonds are extremely sensitive to free-radical mediated oxidation, and it is discolored easily with oxidation of linoleic acid[26]. In the present study, we found that the L.S has a relatively strong effect against the discoloration of β-carotene with linoleic acid.

The mechanism of bleaching of β-carotene is a free radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid by air oxidation. The antioxidant activity of carotenoids is based on the radical adducts of carotenoids with free radical formed from linoleic acid. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation in this model system, in the absence of an antioxidant, the compound loses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically[25].

FRAP assay was performed to estimate the capacity of EELSF to reduce Fe^{3+}/Fe^{2+}. FRAP assay measures the reducing capability by increased sample absorbance based on the formed ferrous ions, and the assay may not be complete even several hours after the reaction starts, such that a single end-point of the reaction cannot be determined[26-30].

Ferric reducing activity based on FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ)[24] (Benzie & Strain, 1996). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom[31].

Finally, the antioxidant activity of EELSF related here brings basis to the traditional use of the plant in different health problems especially for those involving the complex inflammatory process in which ROS and free radicals play a determinant role. Consumers demanding for healthy food products provide an opportunity to develop antioxidants as new functional foods. The result is very much needed by the food industry in order to find possible alternatives to synthetic preservatives. Despite their relative potency in these assays, the consideration of their use as food/ cosmetics-related preservatives or as components for the fictionalization of foods and the formulation of nutraceuticals is a valid one. However, further research would be required before such uses could be proposed with confidence. The findings of the current report appear useful for further research aiming to isolate and identify the specific compounds responsible for the antioxidant activity of EELSF.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors gratefully acknowledge the financial assistance from the University Grants Commission (UGC – File No. 2678/09 (MRP/UGC – SERO), New Delhi, India.

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