IN VITRO ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF *LINUM USITATISSIMUM*

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Summary

The aim of present investigation was to evaluate the *in-vitro* antioxidant activity of ethanolic extract of *Linum usitatissimum* (EE-LU). The methods used were DPPH radical scavenging, reducing power, superoxide anion radical scavenging, hydroxyl radical scavenging, hydrogen peroxide scavenging and metal chelating assay. The doses of EE-LU were 100, 200, 300, 400 and 500 µg/ml respectively. α-tocopherol (100, 200, 300, 400 and 500 µg/ml) was used as a standard antioxidant. The results indicated significant dose dependent inhibition against DPPH radical, reducing power, superoxide anion radical scavenging, hydroxyl radical scavenging, metal chelating and hydrogen peroxide scavenging by EE-LU and α-tocopherol. It is concluded that ethanolic extract of *Linum usitatissimum* (EE-LU) showed dose dependent antioxidant activity, maximum at 500 µg/ml.

Keywords: Antioxidant activity; Flaxseed; Free radical scavenging; *Linum usitatissimum*; Total phenolics.

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Introduction

Antioxidants are vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centred free radicals and other reactive oxygen species (ROS), which are continuously, produced *in vivo*, result in cell death and tissue damage. The role of oxygen radicals has been implicated in several diseases, including cancer, diabetes and cardiovascular diseases, ageing, etc [1]. The most widely used antioxidants, butylated hydroxytoluene and butylated hydroxyanisole have been restricted recently because of serious concerns about their carcinogenic potential and their toxicity and DNA damage induction. Therefore, there is great interest in finding new and safe antioxidants from natural sources [2]. Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense. In this respect flavonoids and polyphenolic compounds have received the greatest attention [3]. Polyphenols possess ideal structural chemistry for free radical scavenging activity and they have been shown to be more effective antioxidants *in-vitro* than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors and from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (termination of the Fenton reaction) [4].

Linum usitatissimum (Linn.), commonly known as flaxseed or linseed belongs to the family Linaceae. Flaxseed is the richest food source of lignan and isoflavonoids[5]. Other compounds reported in flaxseed, which might contribute to therapeutic activity includes hydroxycinnamic acids, ferulic acid and their glycosides, flavonoids herbacetin diglucoside and kaempferol diglucoside [6,7]. In folk medicine, flaxseed is mostly used in the form of tinctures for the treatment of chronic constipation. Flaxseed also enters as a component into some herbal preparations used for the treatment of inflammatory disorders in gynecological practice, as well as for the treatment of anemia, bronchial asthma, stomach cancer, gastritis, diabetes mellitus, hyperthyroidism, prostatic hypertrophy, urethritis, nephrosclerosis etc [8].

The potential health benefits of flaxseed are anticancer effects [9], antiviral, bactericidal [10] and anti-inflammatory activities [11]. Moreover flaxseeds have been reported to be useful in the treatment of diabetes [12,13,14], hypercholesterolemic menopause [15], hypertriglyceridemia and reduction of atherogenic risks [16,17]. Most of the reported biological activities and active constituents of this plant may be related to its antioxidant nature. The objective of present investigation was to study the *in vitro* antioxidant activity of ethanolic extract of *Linum usitatissimum* (called as EE-LU) for free radical scavenging, reducing power, superoxide anion radical scavenging, metal chelating activity, hydroxyl radical scavenging and hydrogen peroxide scavenging activities.

Material and methods

Collection and authentication of plant

Fresh seeds of *Linum usitatissimum* were purchased from local flax supplier of Pune, Maharashtra State, India. The seeds of *Linum usitatissimum* was identified and authenticated by Dr. P. B. Ghorpade, Principal, Scientist and Linseed breeder, All India Co-coordinated Research Project on linseed, College of Agriculture, Nagpur, India and voucher specimen was deposited at the institute.

Drugs and chemicals

1,1-Diphenyl-2-picryl-hydrazyl (DPPH˙), α-tocopherol, 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), thiobarbituric acid (TBA), trichloraceticacid (TCA), potassium ferricyanide, nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), ferric chloride, ascorbic acid and 2-deoxyribose, Folin-Ciocalteau reagent, gallic acid, sodium carbonate, were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hydrochloric acid, methanol, sodium hydroxide, and hydrogen peroxide (H_2O_2) were purchased from Merck (India). Disodium hydrogen phosphate (Research Lab, India), Potassium dihydrogen phosphate (S.D. Fine, Mumbai) were purchased from respective vendors. All other chemicals were of analytical grade.

Preparation ethanolic extract of Linum usitatissimum

The seeds of *Linum usitatissimum* were crushed and milled. These seeds were defatted by petroleum ether (60°- 80° C) in soxhlet apparatus. The marc was then hydrolyzed with 1 M aqueous sodium hydroxide for 1 h at room temperature by constant rotation, followed by extraction with 50% ethanol. Then solution was acidified to pH 2-4 using 1 M hydrochloric acid. The filtrate was dried on tray dryer at 50°C. The yield of the extract was 14.81% w/w. The powdered ethanolic extract was dissolved in distilled water to prepare desired concentration of drug solution and used for *in-vitro* antioxidant study.

Determination of free radical scavenging activity

The scavenging activity of the EE-LU was determined by using 1,1-diphenyl-2-pycrylhydrazyl (DPPH˙) assay previously reported by Bakar et al [18]. About 1 ml of EE-LU solution (100, 200, 300, 400 and 500 µg/ml) and α-tocopherol (100, 200, 300, 400 and 500 µg/ml) were mixed with 5.0 ml of 1 mM (DPPH˙) in absolute methanol. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The absorbance was read by UV-visible spectrophotometer (Jasco V-530, Japan) against methanol at 517 nm. The experiment was repeated triplicate. The activity was expressed as percentage DPPH-scavenging activity relative to the control, using the following equation:

% Inhibition (DPPH˙) = [1-{absorbance of sample/absorbance of control}] × 100

Determination of reducing power

Reducing power of EE-LU was determined by previously reported method of Oyaizu [19]. Briefly, to1 ml EE-LU solution (100, 200, 300, 400 and 500 µg/ml) as well as α-tocopherol (100, 200, 300, 400 and 500 µg/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). The reaction mixture was incubated at 50° C for 20 min. After incubation, 2.5 ml of trichloroacetic acid (10%) was added and centrifuged at 7000 r.p.m. for 10 min. Then 2.5 ml solution from the upper layer was mixed with 2.5 ml distilled water and freshly prepared 0.5 ml $FeCl^3$ (0.1%). The absorbance of sample solutions was read by UV-visible spectrophotometer at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of superoxide anion radical scavenging activity

The method described by Liu et al [20] with modification of Oktay [21] was used for determination of superoxide anion scavenging activity of EE-LU. Superoxide radicals are generated non-enzymatically in PMS–NADH (phenazine methosulphate-nicotinamide adenine dinucleotide) systems by the oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 3 ml of Tris–HCl buffer (16 mM, pH 8.0) containing NBT (50 µM) solution and 1 ml NADH (78 µM) solution and sample solution of the EE-LU (100, 200, 300, 400 and 500 µg/ml) as well as α-tocopherol (100, 200, 300, 400 and 500 µg/ml) in methanol.

The reaction was initiated by adding 1.0 ml of phenazine methosulphate (PMS) solution (10 µM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance was read at 560 nm by UV-visible spectrophotometer. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

% Inhibition (Superoxide anion) = [1-{absorbance of sample/absorbance of control}] × 100

Scavenging of hydrogen peroxide

The ability of the *Linum usitatissimum* to scavenge H_2O_2 was determined according to the method of Ruch et al [22]. Different concentration of EE-LU (100, 200, 300, 400 and 500 µg/ml) and standard α-tocopherol (100, 200, 300, 400 and 500 µg/ml) were added to a H_2O_2 solution (0.6 ml, 40 mM). Absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 of *Linum usitatissimum* and standard compounds was determined by following formula:

% Scavenged [H_2O_2]= [1-{absorbance of sample/absorbance of control}] × 100

Metal chelating activity

The chelation of ferrous ions by EE-LU and α-tocopherol were determined by the method of Dinis et al [23]. EE-LU (100, 200, 300, 400, 500 µg/ml) as well as α-tocopherol (100, 200, 300, 400, 500 µg/ml) were added to a 0.05 ml solution of $FeCl_2$ (2 mM). The reaction was initiated by the addition of 0.2 ml ferrozine (5 mM) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was read by UV-visible spectrophotometer at 562 nm. All tests and analysis were run in triplicate and averaged. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following formula:

% Inhibition (Metal chelating) = [1-{absorbance of sample/absorbance of control}] × 100

Determination of hydroxyl radical scavenging activity

Deoxyribose method of Halliwell et al [24] was used to determine the hydroxyl radical scavenging activity. The reaction mixture, which contained EE-LU (100, 200, 300, 400 and 500 µg/ml) as well as α-tocopherol (100, 200, 300, 400 and 500 µg/ml), deoxyribose (3.75 mM), H_2O_2 (1 mM), potassium phosphate buffer (20 mM, pH 7.4), $FeCl_3$ (0.1 mM), EDTA (0.1 mM) and ascorbic acid (0.1 mM), was incubated in a water bath at 37±0.5 ^{0}C for 1 h. About 1 ml of TBA (1% w/v) and 1 ml of TCA (2.8% w/v) were added to the mixture and heated in a water bath at 100 ^{0}C for 20 min. The absorbance of the resulting solution was measured by UV-visible spectrophotometer at 532 nm. All the analysis was performed in triplicates. The percent inhibition of deoxyribose degradation was calculated by the following formula:

% Inhibition {hydroxyl (OH^-) radical} = [1-{absorbance of sample/absorbance of control}] × 100

Determination of total phenolic content

Total soluble phenolics in the extracts were determined with Folin-Ciocalteau reagent according to the method of Slinkard and Singleton [25] using gallic acid as a standard compound. About 1.0 ml of EE-LU in a volumetric flask was diluted with 46 ml of distilled water, to this 1.0 ml of Folin-Ciocalteau reagent was added and the content of the flask mixed thoroughly. After 3 min 3.0 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking.

The absorbance of the blue color that developed was read at 760 nm. The concentration of total phenols was expressed as mg/g of dry extract. The concentration of total phenolic compounds in the extract was determined as µg of gallic acid equivalent using an equation obtained from the standard gallic acid graph:
Absorbance = 0.005 X gallic acid (µg)

Statistical analysis
All analysis was performed in triplicate. Data was expressed as mean ± S.E.M. Statistical analysis was carried out by one way ANOVA followed by *post hoc* Tuckey test performed using GraphPad InStat version 3.00 for Windows Vista TM BASIC, GraphPad Software, San Diego, California, USA. $P < 0.05$ was considered statistically significant.

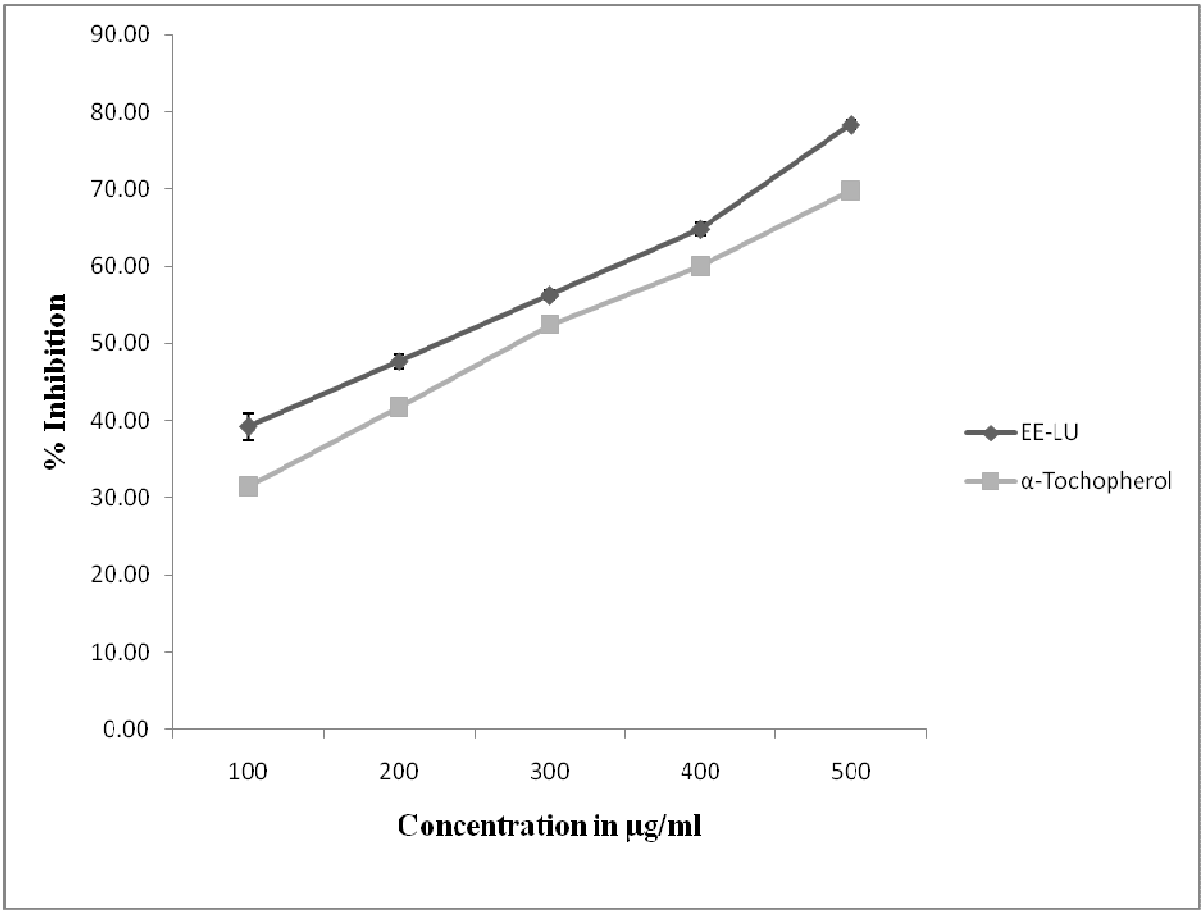
Results and discussion

Determination of free radical scavenging activity
The DPPH˙ scavenging effect of EE-LU was 39.18, 47.61, 56.21, 64.81 and 78.38 % at the concentration of 100, 200, 300, 400 and 500 µg/ml respectively. While DPPH˙ scavenging effect of α-tocopherol was 31.39, 41.71, 52.43, 60.06 and 65.62 % at the concentration of 100, 200, 300, 400, 500 µg/ml respectively. The results thus indicated that free radical scavenging activity increased with increasing concentration (Fig 1).

Free radicals play an important role in chronic diseases related to oxidative stress, such as diabetes, cancer and cardiovascular pathologies. Therefore the free radical scavenging properties of the EE-LU was determined by the DPPH assay. The effect of antioxidants on DPPH radical scavenging was thought to result from their hydrogen donating ability. DPPH˙ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [26]. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH–H by the reaction [27]. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in methyl alcohol was at 517 nm. Antioxidant cause decrease in absorbance of DPPH radical by hydrogen donation which generates as a result of reaction between antioxidant and radical. It is visually noticeable as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants [28]. Scavenging effects of EE-LU on DPPH radicals increased with increased concentrations.

EE-LU and α-tocopherol were capable of scavenging DPPH radicals in a concentration dependent manner . A maximum inhibition value of EE-LU and α-tocopherol was found to be 78.38 and 65.62 percentage respectively at 500 µg/ml. The DPPH radical scavenging activity of EE-LU was significantly ($p<0.01$) more than α-tocopherol at all the concentration used.

Figure 1: Free radical scavenging activity of different concentrations of EE-LU and α-tocopherol by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals.



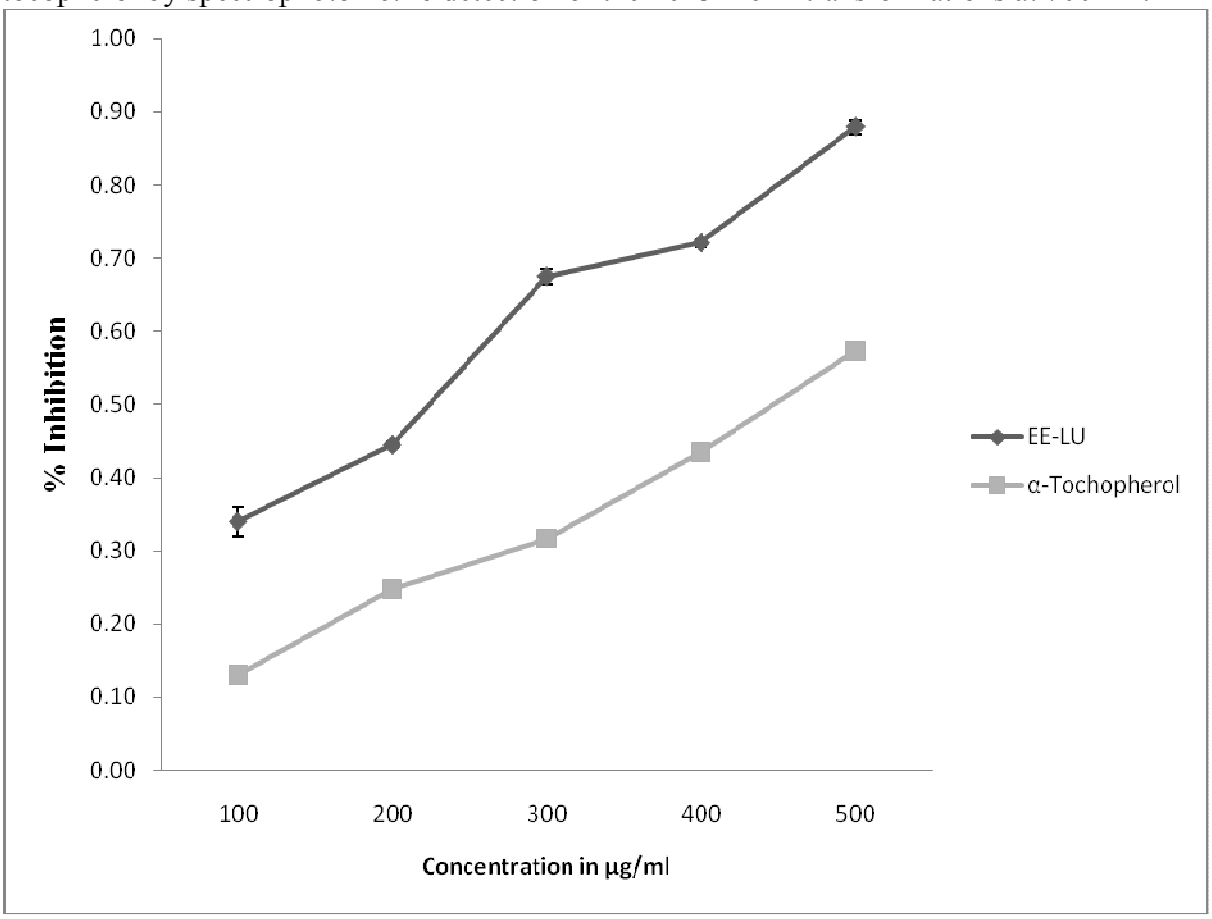
Values are mean ± S.E.M., n=3 in each group; Statistical analysis by one way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; All values were significant *p* value **<0.01.

Determination of reducing power

The absorbance was 0.34, 0.44, 0.67, 0.72 and 0.88 at the concentration 100, 200, 300, 400 and 500 µg/ml of EE-LU respectively. The absorbance by α-tocopherol was 0.13, 0.25, 0.32, 0.44 and 0.57 at the concentration of 100, 200, 300, 400 and 500 µg/ml respectively (Fig-2).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [29]. The antioxidant activity of an antioxidant compound has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [30]. For the measurements of the reductive ability, we investigated the Fe^{3+}-Fe^{2+} transformation in the presence of EE-LU by using the method of Oyaizu [19]. The reducing power of EE-LU was increased with increasing concentration. At the all concentrations, EE-LU showed significantly (p<0.0001) more reducing activity than α-tocopherol.

Figure 2: Comparison of reducing power of different concentrations of EE-LU and α-tocopherol by spectrophotometric detection of the Fe+3–Fe+2 transformations at 700 nm.



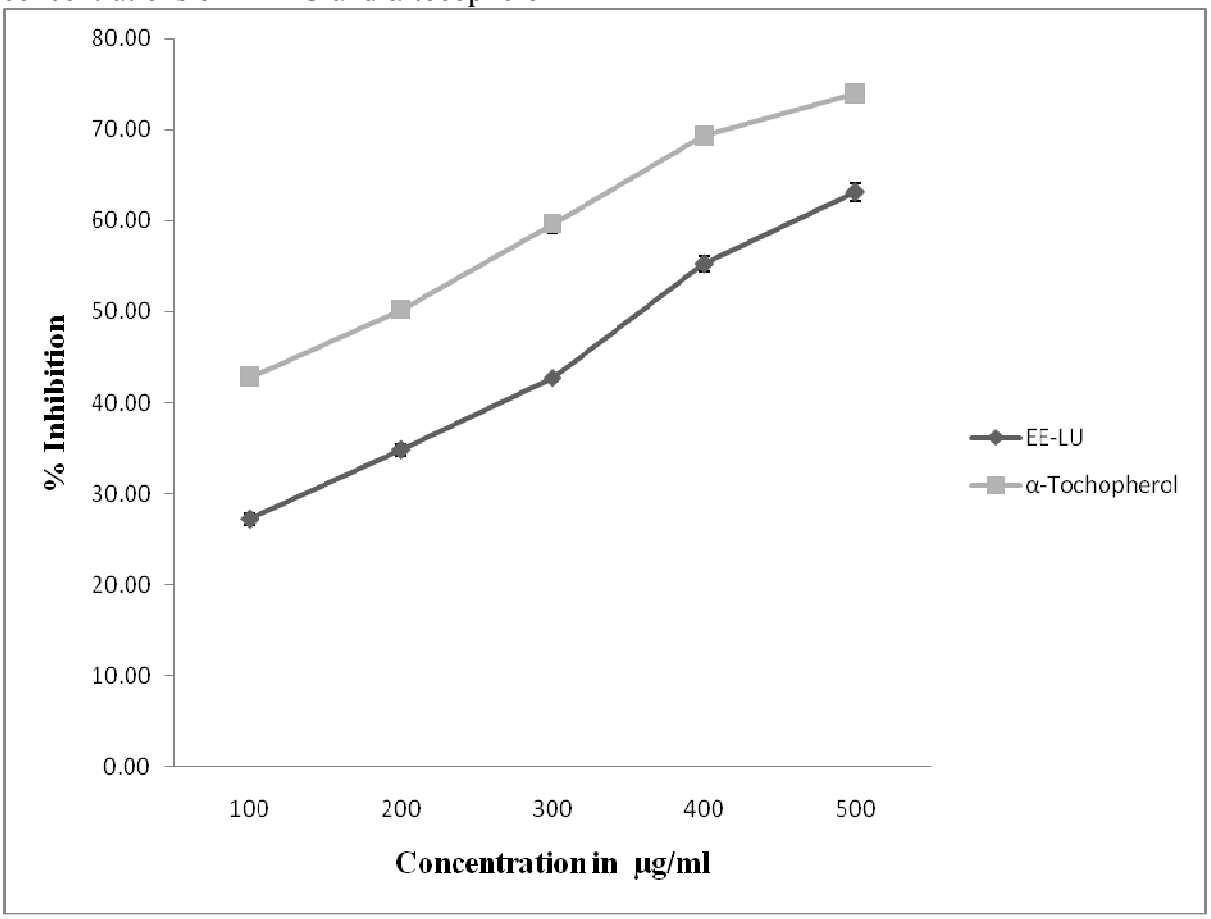
Values are mean ± S.E.M., n=3 in each group; Statistical analysis by one way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; All values were significant *p* value ***<0.0001.

Determination of superoxide anion radical scavenging activity

The percentage inhibition of superoxide radical generation by EE-LU at 100, 200, 300, 400 and 500 µg/ml was 27.19, 34.81, 42.67, 55.22 and 63.12 % respectively whereas α-tocopherol had 42.77, 50.21, 59.64, 69.28 and 73.97 % respectively inhibition by the at concentration 100, 200, 300, 400 and 500 µg/ml respectively (Fig. 3). The percentage inhibition of superoxide radical generation by EE-LU was significantly (p<0.0001) less than that of shown by α-tocopherol, indicating weak antioxidant effect in this test.

In the early nineties, Prior and Cao [31] developed an assay called the oxygen radical absorbance capacity (ORAC) to quantify the antioxidant capacity of a number of products including fruits and vegetables. In this study, we have used PMS/NADH-NBT system; in which superoxide anions are derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.

Figure 3: Comparison of superoxide anion radical scavenging activity of different concentrations of EE-LU and α-tocopherol



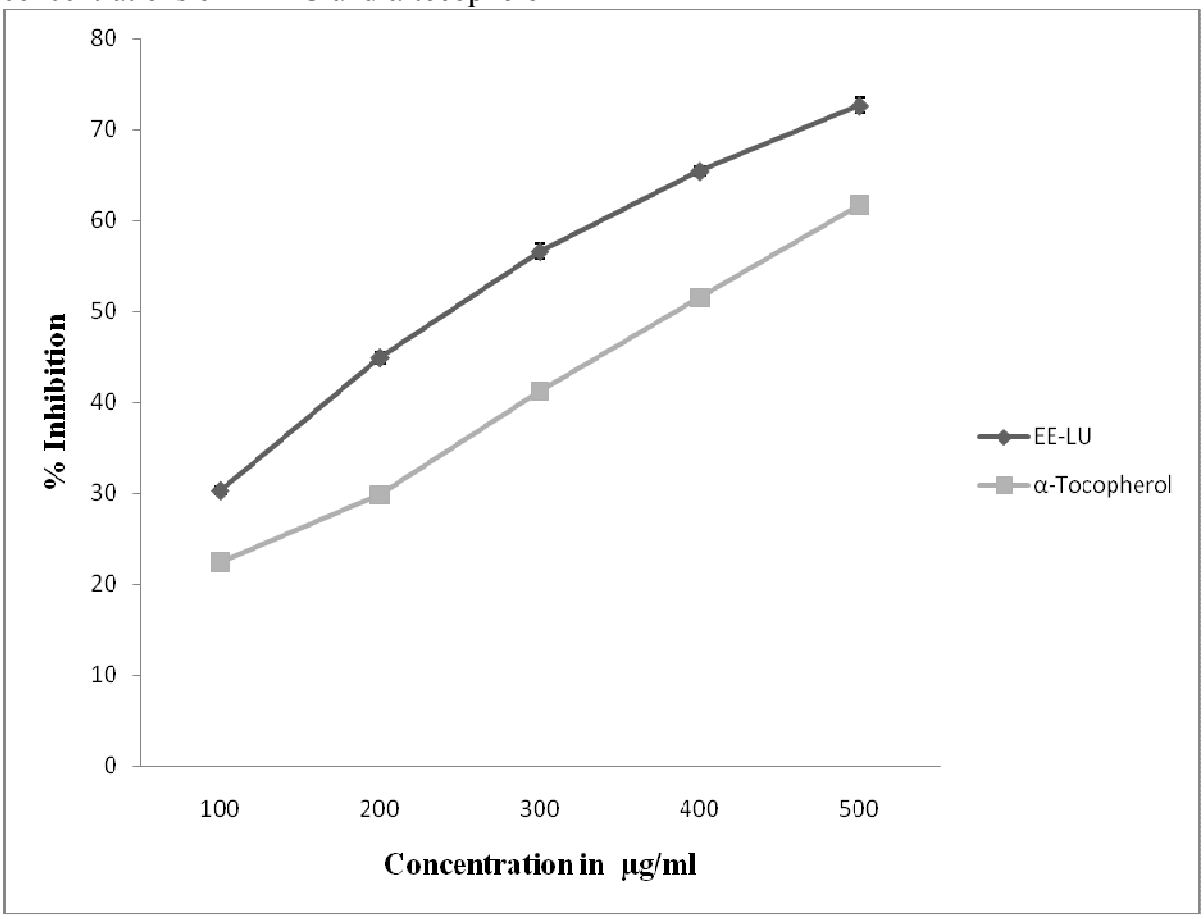
Values are mean ± S.E.M., n=3 in each group; Statistical analysis by one way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; All values were significant *p* value ***<0.0001.

Scavenging of hydrogen peroxide

The hydrogen peroxide radical scavenging activity of EE-LU was 30.27, 44.90, 56.61, 65.43 and 72.64 % at the concentration of 100, 200, 300, 400 and 500 µg/ml respectively. While hydrogen peroxide scavenging activity of α-tocopherol was 22.35, 29.76, 41.18, 51.46 and 61.68% at the concentration of 100, 200, 300, 400 and 500 µg/ml respectively (Fig. 4).

The percentage H_2O_2 scavenging effect by the same concentration (500 µg/ml) of EE-LU and α-tocopherol was found as 72.64 and 61.68 respectively. It is thus apparent that EE-LU was more effective (p<0.0001) than α-tocopherol with respect to hydrogen peroxide scavenging activity. H_2O_2 is highly important because of its ability of penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be celltoxic because it may give rise to hydroxyl radical in the cells. Thus, removing H_2O_2 is very important for the protection of living systems [26].

Figure 4: Comparison hydrogen peroxide radical scavenging activity of different concentrations of EE-LU and α-tocopherol



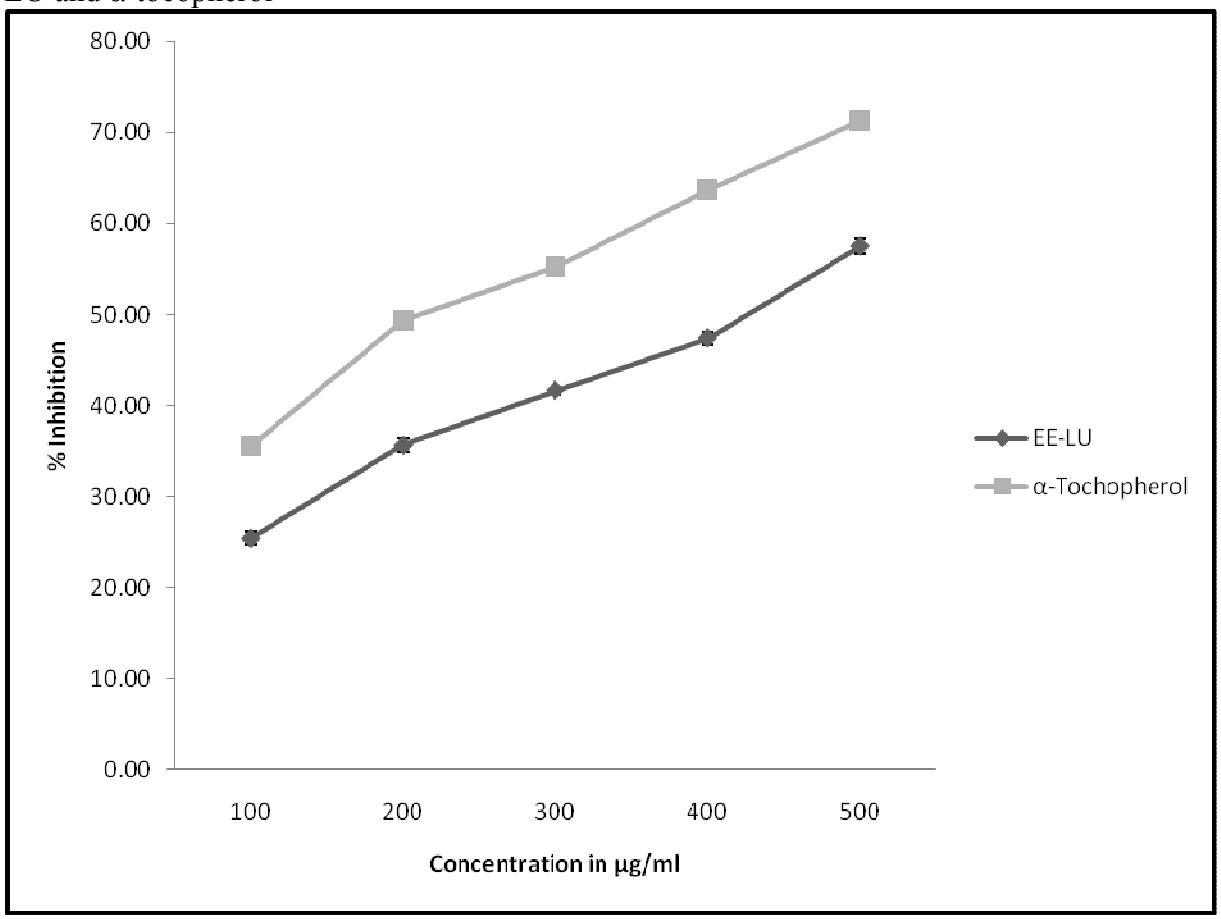
Values are mean ± S.E.M., n=3 in each group; Statistical analysis by one way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; All values were significant *p* value ***<0.0001.

Metal chelating activity

The percentage of metal chelating capacity of 100, 200, 300, 400 and 500 µg/ml of EE-LU was 25.39, 35.62, 41.66, 47.40 and 57.51 % respectively. While metal chelating capacity of 100, 200, 300, 400 and 500 µg/ml of α-tocopherol was 35.57, 49.36, 55.32, 63.70 and 71.36 % respectively (Fig. 5). The chelating of ferrous ions by EE-LU was estimated by the method of Dinis et al [23]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of color reduction, therefore allows estimation of the chelating activity of the coexisting chelator [32]. In this assay EE-LU and standard α-tocopherol interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine. Iron can stimulate lipid peroxidation by fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation [33]. The formation of the Fe^{2+}-ferrozine complex is not complete in the presence of EE-LU, indicating that EE-LU chelates iron. The absorbance of Fe^{2+}-ferrozine complex was linearly decreased in a dose dependent manner. The mean percentage of metal chelating capacity of EE-LU and α-tocopherol were found to be 44.12 and 64.12 respectively. Metal chelating capacity is significant since it reduced the concentration of the catalysing transition metal in lipid peroxidation.

It was reported that chelating agents, which forms σ-bonds with metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [26]. The data obtained reveal that the EE-LU demonstrated significantly (p<0.0001) more capacity for iron binding, than α-tocopherol and thereby better peroxidation inhibition.

Figure 5: Comparison metal chelating scavenging activity of different concentrations of EE-LU and α-tocopherol



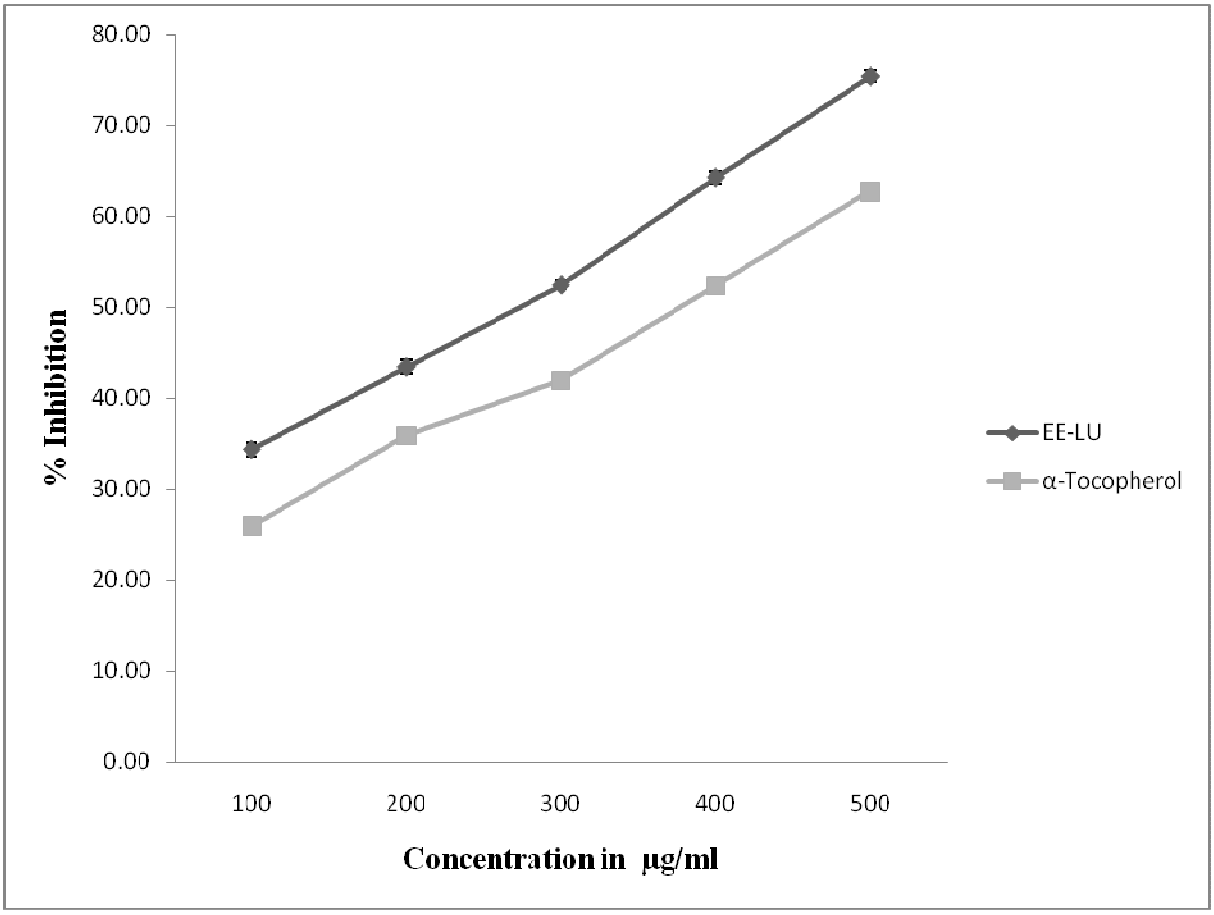
Values are mean ± S.E.M., n=3 in each group; Statistical analysis by one way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; All values were significant p value ***<0.0001.

Determination of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of EE-LU was 34.36, 43.36, 52.46, 64.27 and 75.38 % at the concentration of 100, 200, 300, 400 and 500 µg/ml respectively. While hydroxyl radical scavenging activity of α-tocopherol was 25.88, 35.87, 41.91, 52.38 and 62.79 % at the concentration of 100, 200, 300, 400 and 500 µg/ml respectively (Fig. 6).

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins [34]. The effect of EE-LU on the inhibition of free radical-mediated deoxyribose damage was assessed by means of the Iron (II)-dependent DNA damage assay. The fenton reaction generates hydroxyl radicals (OH), which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. The EE-LU was significantly (p<0.001) more effective in reducing DNA damage at all concentrations compared to α-tocopherol.

Figure 6: Comparison of hydroxyl radical scavenging activity of different concentrations of EE-LU and α-tocopherol



Values are mean ± S.E.M., n=3 in each group; Statistical analysis by one way ANOVA followed by post hoc Tukey's test using Graphpad Instat software; All values were significant *p* value ***<0.001.

Determination of total phenolic content

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Contents of flavonoid and other phenolic substance have been suggested to play a preventive role in the development of cancer and heart disease [35]. Phenolic compounds are attracting considerable interest in the fields of food chemistry and medicine due to their promising antioxidant potential [36]. In the present study, the Folin–Ciocalteu method was used to determine the total phenolic compounds. In EE-LU, 32.26 mg gallic acid equivalent of phenols was detected. Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups [37]. The phenolic compounds may contribute directly to antioxidative action [38]. Phenolic compounds are one of the most widely distributed secondary plant products. The ability of these compounds to act as antioxidant has been well established. Polyphenols are multifunctional by acting as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers [39]. Presence of low concentration of polyphenols onto substrate is essential to delay retard or prevent autooxidation or free radical mediated oxidative process.

It is apparent that EE-LU showed more DPPH radical scavenging activity, reducing power, hydroxyl radical scavenging and hydrogen peroxide radical scavenging but less superoxide scavenging and metal chelation activity than α-tocopherol. Phenolic compounds seem to be the main components responsible for the antioxidant activity.

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