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Evaluation of antioxidant potential and qualitative analysis of major polyphenols by RP-HPLC in *Luffa acutangula* var. *amara* Roxb. pericarp extracts

Mohan G. Kalaskar*, Anil U. Tatiya and Sanjay J. Surana

R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, Dhule, MS, India

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ABSTRACT

The present study was designed to identify antioxidant potential and phenolic content of Luffa acutangula Var. amara fruit pericarp extracts. The antioxidant activity of the extracts were assessed by using various in-vitro tests such as DPPH, ABTS, superoxides radical, reducing power and phosphomolybdenum assay. Among the all extracts in present study, the ethanolic extract of fruit pericarp produced potent antioxidant activity and showed presence of gallic acid and catechin as confirmed by HPLC. Further, total phenolic and flavonoid contents were also found to have positive correlation with antioxidant potential of the extract. The results of present study clearly indicated that the L. amara fruit pericarp can be used as potential source of natural antioxidant.

Keywords: *Luffa acutangula*, Antioxidant activity; Free radicals; DPPH, HPLC, fruit pericarp.

INTRODUCTION

The free radicals are continuously produced in living organisms as a result of biochemical reactions, are fundamental in modulating various physiological functions and represent an essential part of aerobic life [1]. The generation of free radicals is regulated by endogenous antioxidant systems. Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radicals [2]. However, the imbalance between free radicals generation and antioxidant defense leads to oxidative stress. The oxidative stress has been implicated to variety of disorders such as neurogenerative, cardiovascular, metabolic etc [3].

It has been observed that natural antioxidants are safer than synthetic antioxidants [4]. Therefore, there is an increasing interest amongst scientific communities in identifying natural source of antioxidants derived from fruits and vegetables. These effects of natural antioxidants are because of phytoconstituents viz, polyphenols, vitamins and carotenoids, which might help to prevent oxidative damage [5, 6]. Traditionally practiced natural antioxidants are already exploited commercially, but still there is demand to find more plant species concerning the antioxidant potential [7].

Luffa acutangula var. *amara* Roxb.(Cucurbitaceae) is a climbing shrub. It's aerial parts are used traditionally in diverse health ailments like jaundice, asthma, piles and emetic [8] and occasionally used in various folklore recipes by tribes of Satpuda region, Maharashtra. In Ayurveda, the plant is used to treat liver complaints, inflammation, tumours, uterine, vaginal tumours and spleenic enlargements [9]. *L. amara* fruit is reported to posses bitter principle luffin, colocynthin, and triterpenoids amarinin [10]. The fruit is reported to be CNS- depressant [11].

Despite these diverse medicinal uses of the plant in the codified and non-codified Indian medicinal systems, the plant has received little investigational coverage. Therefore, the present study was undertaken to antioxidant potential of various extracts of *L. amara* fruit pericarp through battery of *in vitro* anti-oxidant tests and it's correlation with polyphenolic content.

MATERIALS AND METHODS

Chemicals

Folin Ciocalteu reagent, Vanillin (Merck, Mumbai.); 2, 2, Diphenyl-1-Picryl hydrazyl (DPPH), Quercetin, Catechin and 2, 2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), (Sigma-Aldrich Chemie, Steinheim, Germany); Ethylene Diamine Tetra Acetic acid, Ascorbic acid (SD Fine chemicals, Mumbai); Dimethyl sulfoxide (DMSO), Ascorbic acid, Ammonium molybdate, Nitro blue tetrazolium (NBT), Riboflavin and Gallic acid (Loba Chemie, Mumbai); Sodium sulphate, Potassium persulfate, Sodium carbonate, Aluminum nitrate, Sodium acetate, All other reagents and solvents were used of analytical grade.

Plant material

The fruits of *L. amara* were collected from Shirpur, Dhule District, Maharashtra, India. Plant specimen was authenticated by Prof. D. A. Patil, Dept. of Botany, SSVP College, Dhule, India. A voucher specimen has been deposited in the herbarium of our laboratory under the number (PCO/LA01).

Extract preparation

The fruits of uniform size (10-12 cm) were collected, cleaned and dried in shade. The seeds were separated from pericarp, pulverized and used for solvent extraction. The 1000 g of pericarp powder was extracted successively with petroleum ether (60-80°), ethyl acetate and ethanol by hot continuous percolation. The resultant marc was macerated with water twice at 25°C for 48 h. extracts after filtration were dried using a rotary evaporator (BUCHI, Rotavapor R - 215) under reduced pressure. The dry extract obtained with each solvent was weighed and percentage yield was expressed in terms of air dried weight of plant material.

Determination of total phenolics

Total phenolics were determined by the spectrophotometric method with slight modification [12] (Singleton & Rossi, 1965). In brief, a 0.1 ml of appropriately diluted extracts was added to 0.2 ml of 10-fold diluted Folin–Ciocalteau reagent. 2.0 ml of 15% sodium carbonate was added to mixture and then shaken. After 2 h incubation period, the absorbance of the reaction mixtures was measured at 760 nm. The standard curve for total phenolics was plotted using gallic acid standard solution (10–100 µg/ml) following the same procedure as mentioned above. The total phenolics were expressed as milligram of gallic acid equivalents (GAE) per gram of dried extract.

Determination of total flavonoids

The total flavonoids were determined according to method described by Liu et al [13]. 10 mg of extracts were dissolved in 100 ml of methanol and filtered. 0.5 ml of diluted extract, 1.5 ml of ethanol, 0.1 ml of 10% aluminum nitrate, 0.1 ml of 1 M sodium acetate and 2.8 ml of water were added and mixed. After 40 min, mixture was measured at 415 nm. The standard curve for total flavonoids was made using quercetin standard solution (10-100 µg/ml) under the same procedure as above. The total flavonoid was expressed as mg quercetin equivalent (QE) per gram of dried extract.

Antioxidant assays

Each extract was dissolved in methanol or DMSO at a concentration 1 mg/ml and further diluted to prepare the series of concentrations. Reference standard was used for comparison in all assays.

DPPH radical-scavenging activity

Radical scavenging activity of plant extracts against stable DPPH was done according to the method of Ebrahimzadeh et al. with minor modifications [14]. Different concentrations of each extracts were added, to an equal volume, methanolic DPPH (100mM) solution. Each of the extract or the reference standard solution was added separately in wells of the microtitre plate. After 20 min at room temperature, the absorbance was measured at 517 nm using Microplate spectrophotometer (BIO-Tek, USA. Model-96 well micro plate). Same procedure was followed for control by using methanol in place of extract. The percentage inhibition was estimated based on the percentage of DPPH radical scavenged using the following formula:

$$\% \text{ inhibition} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

ABTS radical cation scavenging activity

ABTS radical cation scavenging activity was performed using the method reported by Fellegrin, Ke, Yang, and Rice-Evans with slight modifications [15]. In brief, ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept overnight in the dark to yield a dark colored solution containing $ABTS^{\cdot+}$ radical cation. Prior to use in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.700 at 734 nm. After the addition of 1.0 ml of diluted $ABTS^{\cdot+}$ to 10 µl of sample, the absorbance was measured after 5 min of initial mixing. The percentage inhibition was calculated according to the formula:

$$\% \text{ inhibition} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

The antioxidant potential of extract was expressed as IC_{50}, the concentration necessary for a 50% reduction of $ABTS^{\cdot+}$ radicals.

Total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of *L. amara* pericarp extracts were evaluated as reported by Saleh and Hameed [16]. An aliquot of 100 µl of extract solutions was combined with 1 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). All tubes were capped and incubated in a boiling water bath at 95^{o}C for 90 min. Tubes were allowed to cool at room temperature. Absorbance of the test and standard solutions was measured at 695 nm against blank containing 0.1 ml of distilled water and 1 ml of reagent. The standard curve for total antioxidant capacity was plotted using ascorbic acid standard solution (20-100 µg/ml) following said procedure. An antioxidant capacity was expressed as millimolar equivalents of ascorbic acid.

Superoxide radical-scavenging activity by Riboflavin-Light-NBT System

The super oxide free radical scavenging activity was carried out as per Bafna and Misra with slight modification [17]. The 200 µl of EDTA, 100 µl of NBT, 50 µl of riboflavin, 2.5 ml of phosphate buffer pH 8.0 and 200 µl of varying concentration of extracts and reference standard were mixed in test tube. Reaction commenced by illuminating the reaction mixture for 15 minutes using fluorescent lamp. After illumination, the absorbance was measured at 590 nm. Same procedure was followed for control by replacing methanol in place of samples. Ascorbic acid was used as standard. The percent inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Reducing power assay

A reducing power assay was pursued by method of Oyaizu [18]. 2.5 ml of extract solution, 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1% w/v) were mixed, and incubated at 50^{o}C for 20 min. 2.5 ml of trichloroacetic acid (10% w/v) was added to each test tube and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of water and 0.5 ml of ferric chloride (0.1% w/v) and absorbance was measured at 700 nm. Increase absorbance of the reaction mixture indicated high reducing power. The IC_{50} was determined (the concentration at 0.5 absorbance).

HPLC analysis

Presence of phenolic compounds in ethanol extract of *L. amara* fruit pericarp were identified by using, Shimadzu class LC-10AT HPLC (The Luna C_{18} reverse-phase column, 250 x 4.6 mm, i.d. particle size 5µm). The output signal was monitored and processed using chromquest version 3.0 software. The detection of Gallic acid (GA) and catechin (CT) was carried out at 280 nm using UV detector (Spectra system UV1000). The solvent system used for GA and CT was 0.1 % H_3PO_4: Acetonitrile (85:15), and Acetonitrile: Water (80:20) with flow rate 0.7, 0.3 ml/min at 25^{o}C, respectively. Identification of the phenolic compounds was carried out by comparing the retention time t those of standard. The analysis was done in triplicates.

Statistical analysis

Descriptive statistical analysis was performed using Microsoft Excel. The data were expressed as mean for samples having triplicate analysis ± SEM. Pearson correlation coefficient of phenolic, flavonoids with antioxidant assays were tested for significance.

RESULTS

The percent extractive yield of successive extracts of *L. amara* fruit pericarp ranges from 0.97 ± 0.15 to 9.32 ± 0.87%, with descending order of aqueous > ethanol > petroleum ether > ethyl acetate extract. An extraction ability of petroleum ether and ethyl acetate extracts is almost same.

Total phenolic content of extract varied between 3.85 ± 0.003 to 30.11 ± 0.005 mg/g GAE.

The highest total phenolic content was recorded in ethanol extract 30.11 ± 0.005 mg GAE, while least in petroleum ether extract 3.85 ± 0.003 mg GAE. The total flavonoid content varied between 5.07 ± 0.001 to 86.50 ± 0.074 mg /g QE of dry extract. The highest flavonoid content was observed in ethyl acetate extract 86.50 ± 0.074, while least was observed in petroleum ether extract 5.07 ± 0.001, as given in Table 1.

In DPPH radical-scavenging assay, radical-scavenging activity of the extracts from fruit pericarp of *L. amara* was estimated by comparing IC_{50} of the extracts and those of ascorbic acid (Table 2).

Table 1. The extractive yields, total phenolic (gallic acid equivalent) and total flavonoid (as quercetin equivalent) compound in extracts of *Luffa amara* Fruit pericarp

Plant extracts	Total phenolics (mg/g DW)	Total flavonoids (mg/g DW)	Extract yield (%W/W)
Pet ether (60-80°)	3.85 ± 0.003	5.07 ± 0.001	1.03 ± 0.11
Ethyl acetate	21.26 ± 0.008	86.50 ± 0.074	0.97 ± 0.15
Ethanol	30.11 ± 0.005	73.64 ± 0.011	1.56 ± 0.25
Aqueous	21.42 ± 0.004	19.34 ± 0.005	9.32 ± 0.87

Each value in the table is represented as mean ±SEM (n = 3)

The observed differential scavenging activities of the extracts against the DPPH system could be due to the presence of different compounds in the extracts. The IC_{50} value for ethanol extract was 84.00 ± 0.76, while petroleum ether extract had IC_{50} of 474.28 ± 1.75. The IC_{50} of ascorbic acid was found be 41.89 ± 0.36. A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value. The scavenging ability of DPPH was in descending order from ethanol > ethyl acetate > aqueous > petroleum ether extract.

Table 2. Antioxidant effect (IC_{50}) on free DPPH radicals, superoxide radicals, ABTS radicals, reducing power assay and total antioxidant capacity of extracts of *Luffa amara* fruit pericarp

Plant extracts	IC_{50} µg/ml				Total antioxidant capacity (AAE/DW)
	Scavenging ability on DPPH radicals	Scavenging ability on super oxide	Scavenging ability on ABTS radicals	Reducing power assay	
Pet ether (60-80°)	474.28 ± 1.75	319.79 ± 0.55	56.76 ± 0.15	866.58 ± 0.39	13.22 ± 0.50
Ethyl acetate	232.02 ± 0.84	75.23 ± 0.43	46.00 ± 0.30	487.51 ± 1.88	28.22 ± 0.37
Ethanol	84.00 ± 0.76	77.69 ± 0.06	43.76 ± 0.62	245.14 ± 0.94	30.72 ± 0.73
Aqueous	414.83 ± 2.56	109.18 ± 1.20	49.26 ± 0.32	821.65 ± 1.21	20.72 ± 0.37
Ascorbic acid	41.89 ± 0.36	20.72 ± 0.07	12.16 ± 0.04	42.41 ± 0.47	---

Each value in the table is represented as mean ±SEM (n = 3)

All extracts from fruit pericarp of *L. amara* exhibited ABTS radical-scavenging activities to different extents in a concentration- dependent manner; although the activity levels of all of the tested samples were lower than that of ascorbic acid. Among all extracts, ethanol and ethyl acetate extract from *L. amara* fruit pericarp exhibited the highest ABTS radical scavenging activity (IC_{50}) i.e. 43.76 ± 0.62 and 46.00 ± 0.30 respectively. In contrast, petroleum ether extract showed least ABTS radical scavenging capacity i.e. 56.76 ± 0.15. The similar result was produced by ascorbic acid nearly at concentration 12.16 ± 0.04 µg/ml (Table 2). The ABTS radical scavenging ability of samples can be ranked as ethanol > ethyl acetate > aqueous > petroleum ether extract.

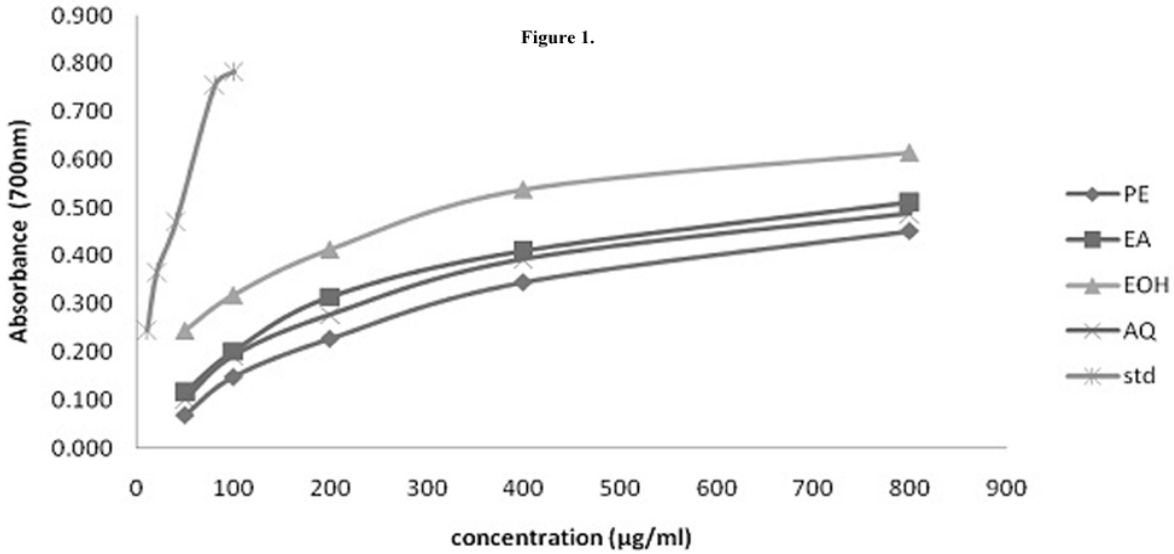
The IC_{50} values in superoxide scavenging activity were found in descending in order as ethyl acetate < ethanol < aqueous < petroleum ether extract. The ethyl acetate extract showed least IC_{50} i.e. 75.23 ± 0.43, while petroleum ether showed the highest IC_{50} i.e. 319.79 ± 0.55 (Table 2). All the extracts showed concentration dependant superoxide radical scavenging activity.

However, when compared to ascorbic acid, the superoxide scavenging activity of the extracts was found to be low. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other phytoconstituents in the extract. The decrease in absorbance at 590 nm with plant extracts and reference standard ascorbic acid indicates their ability to quench superoxide radical in reaction mixture. It may be due to presence of flavonoid and phenolic content.

The total antioxidant capacity of *L. amara* extracts was determined with reference to ascorbic acid. Total antioxidant capacity for ethanol and ethyl acetate extract were 30.72 ± 0.73 and 28.22 ± 0.37 mg of ascorbic acid equivalent/g of dry extract (AAE) respectively. While petroleum ether extract had less total antioxidant capacity i.e. 13.22 ± 0.50 mg AAE (Table 2). The total antioxidant capacity of different extracts can be ranked in descending order as: ethanol > ethyl acetate > aqueous > petroleum ether extracts.

Figure 1 shows the dose-response curves for the reducing powers of the extracts from *L. amara* fruit pericarp. The reducing power of the ethyl acetate and ethanol extract increased in absorbance from 0.245 ± 0.006 and 0.116 ± 0.001 at 50 µg/ml, to 0.615 ± 0.004 and 0.512 ± 0.004 at 800 µg/ml, respectively. The reducing power of aqueous and petroleum ether extracts increased from 0.101 ± 0.001 and 0.068 ± 0.004, at 50 µg/ml l to 0.488 ± 0.003 at 0.451 ± 0.006 at 800 µg/ml, respectively. At a dosage of 400 to 800 µg/ml extracts showed high reducing values of 0.345 – 0.615, almost equal to that of ascorbic acid (0.472 -0.754) at a concentration of 40 - 80 µg/ml.

Figure 1. Reducing power of extracts from *Luffa amara* fruits. The Ascorbic acid is used as reference antioxidant. Values are mean ± SEM (*n* = 3)



**PE – Petroleum ether (60-80°) extract, EA – Ethyl acetate extract, EOH – Ethanol Extract, AQ – Aqueous extract, Std - satandard.*

The IC_{50} value was very high for the petroleum ether and aqueous extract (866.58 ± 0.39 µg/ml and 821.65± 1.21 respectively), compared with ethyl acetate and ethanol extracts, of which the IC_{50} values were 245.14 ± 0.94 and 487.51 ± 1.88 µg/ml, respectively. An IC_{50} value of ascorbic acid was 42.41 ± 0.47 µg/ml. The sequence for reducing power capacity was ethyl acetate > ethanol > aqueous > petroleum ether.

The correlation analysis for IC_{50} values of radical scavenging and or antioxidant ability of extract of *L. amara* pericarp, contents of phenolics and flavonoids exhibited good correlation with DPPH, ABTS, total antioxidant capacity, superoxide radicals and reducing power assay (Table 3).

Table 3. Correlations[A] between the IC_{50} values of antioxidant activities and polyphenolic content of *L. amara* fruit pericarp

Assay	Correlation R^2	
	Phenolics	Flavonoids
IC_{50} of DPPH radical scavenging ability	0.7007[b]	0.7879[b]
IC_{50} of Total antioxidant capacity	0.6963[b]	0.9380[a]
IC_{50} of ABTS radical cation scavenging activity	0.9402[a]	0.7534[b]
IC_{50} of Super Oxide Free Radical Scavenging Activity	0.8835[b]	0.6000[b]
IC_{50} of Reducing power assay	0.6304[b]	0.7850[b]

[A] L. amara fruit extracts were used in correlation; [a] Indicate significance at p > 0.05; [b] Indicate significance at p < 0.05

The R^2 value above 0.5 is considered good correlation. Total phenol content was shown to provide the highest association with ABTS radical scavenging activity in the present study (R^2 = 0.9402). Similar results were also found for super oxide free radical scavenging activity (R^2 = 0.8835). While total flavonoid content shown good correlation with Total antioxidant capacity (R^2 = 0.9380).

DISCUSSION

The relation between diseases and free radicals has been proved by many studies. UV light, radiation, smoking, alcohol consumption, stress and high cholesterol consumption can increase the process of cell oxidation [19]. The present study was aimed to establish antioxidant capacity and their correlation with polyphenolic content of fruits. The polyphenolic compounds which contain hydroxyl groups in their structure and their electron donating ability which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers and responsible for antioxidant property [20]. The phenolic compounds may contribute directly to antioxidative action[21]. It is known that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans [22]. The total flavonoids and phenolic contents in ethanol and ethyl acetate fractions were significantly higher than petroleum ether and water extracts. Therefore, it can be presumed that the major polyphenolic compounds present in these extracts.

The DPPH and ABTS radicals are soluble in water and organic solvent, thus enabling the determination of antioxidant capacity of both hydrophilic and lipophillic compounds [23]. The DPPH and ABTS radical scavenging activity of the ethanol extract revealed high antioxidant activity, the possible reason might be the different contents and sorts of bioactive compounds including phenolics and other compounds responsible for antioxidant capacity. These results were consistent with the findings of many research groups, who reported such correlations between total phenolic content and free-radical scavenging activity [24, 25].

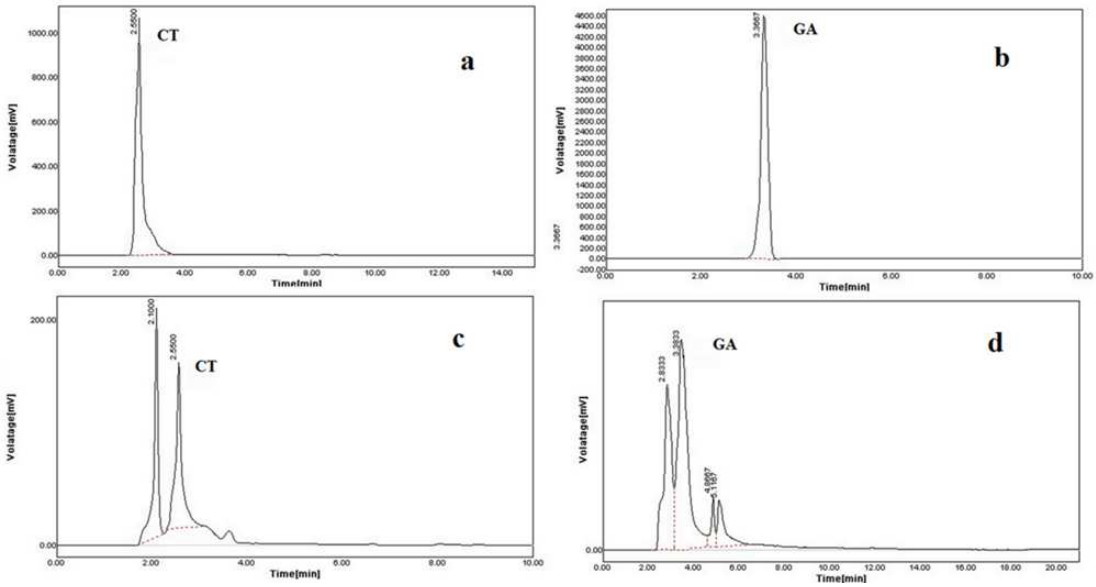
Superoxide radical is very harmful cellular component, it can generate more ROS. These can cause tissue or DNA damage leads to various diseases, therefore it is recommended to measure comparative interceptive ability of antioxidant extract to scavenge superoxide molecule[26]. In present study, superoxide radical reduces NBT to blue colored formosan that is measured at 590 nm. The result shows that ethanol and ethyl acetate extracts has potent scavenging activity that reveled form low IC_{50}. The probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of extracts towards generation of superoxide in the *in vitro* reaction mixture.

The phosphomolybdenum assay is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity). The ethanol extract demonstrated electron-donating capacity showing its ability to reduce MO (VI) to MO (V) and forms a green colored phosphomolybdenum V complex and act as chain terminators. Thus, transforming relative free radical species into more stable non-reactive products. The results obtained in this investigation reveal that the total antioxidant activity may be attributed to the presence of phenolics and flavonoids constituents in ethanol extracts [27].

In reducing power assay, ethanol extract of fruit pericarp were capable of chelating ferrous ion effectively, which indicated that the ethanol soluble compounds of fruit pericarp was active for ferrous ion chelating. Transition metals are known to play key roles in lipid peroxidation in both biological and food system. Particularly, reaction of ferrous iron with hydrogen peroxide generates the hydroxyl radicals, which are the most reactive and detrimental reactive oxygen species in any biological system. They can subsequently oxidise the surrounding biomolecules. Therefore, ferrous ion is considered as the most effective pro-oxidant in food and biological systems. It has been well established that lipid peroxidation can be suppressed by chelating agents stabilizing transition metals to inhibit the production of free radicals [28].

Figure 2. HPLC chromatogram of (a) authentic standards catechin (CT), (b) gallic acid (GA), (c) *catechin and* (d) gallic acid was identified in ethanol extract of *L. amara* fruit pericarp

Figure 2.



Furthermore, all *in vitro* antioxidant assay shown good correlation with total phenolic and flavonoid content of extracts, which indicates the antioxidant activity of fruit pericarp is due to polyphenolic compounds. The ethanol extract showed most potent antioxidant activity, because of presence of phenolic compounds, particularly flavonoids, hydrolysable and condensed tannins due to the presence of the hydroxyl groups. The presence of catechin and gallic acid in ethanol extract of fruit pericarp was chromatographically confirmed. Catechin and gallic acid has a definite antioxidant potential mainly due to the strong reducing power and weak metal chelating ability and could have a role as a physiological antioxidant [29, 30]. Perhaps catechin, gallic acid and other related phenolic compounds present in *L. amara* fruit pericarp may be responsible for its observed antioxidant activity. Further studies on the in vivo evidence of pro-oxidant activity of *L. amara* fruits are required.

CONCLUSION

The present investigation reveals that ethanol extracts of *L. amara* fruits exhibit high antioxidant capability. This activity is attributed to high levels of total phenolic and flavonoid compounds particular to gallic acid and catechin. Consequently, our results suggest that the extract can be utilized as an effective and safe antioxidant source, although the antioxidant activities of ethanol extract was lower than that of ascorbic acid. It can be concluded that, fruits of *L. amara* used in Ayurveda and folklore practice for variety of diseases, can be used as an accessible source of natural antioxidants with consequent health benefits. Further scientific work is needed to ensure the other medicinal properties of the plant in correlation to antioxidant activity.

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