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ORIGINAL ARTICLE

Free radical scavenging activity of methanolic extract of *Luffa cylindrica* leaves

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Context: Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, hypertension, arthritis, ischemia, gastritis, central nervous system injury, reperfusion injury of many tissues, cancer, Alzheimer's disease, Parkinsonism, diabetes mellitus and AIDS. There is considerable evidence that antioxidants could help to prevent these diseases because they have the capacity to quench free radicals. **Aim:** Free radical scavenging activity of methanolic extract of the leaves of *Luffa cylindrica* (MELC) was evaluated in various *in vitro* systems. **Materials and Methods:** The methods were extensively reviewed and free radical scavenging activity was performed by employing various *in-vitro* assay methods like DPPH, hydroxyl radical, superoxide and nitric oxide scavenging activities. **Statistical Analysis:** Results were analyzed statistically using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and were expressed as mean ± SE of three observations. Values of $P < 0.05$ were considered significant. **Results:** In all the studies, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals. The preliminary phytochemical screening of MELC indicated the presence of terpenoids, steroids, flavonoids and glycosides. The extract was found to contain 53.78 ± 1.01 µg/mg total polyphenolics expressed as GAE (micrograms per milligram of GAE). **Conclusion:** The results of the study suggested that the methanolic extract of the leaves of *Luffa cylindrica* possessed a significant scavenging effect with increasing concentrations probably due to its antioxidant potential and could serve as a potential source of natural antioxidants effective in treatments against free radical mediated diseases.

Key words: Antioxidant, *Luffa cylindrica*, methanolic extract, phenolic compounds

INTRODUCTION

Oxidation is well known to be major cause of foods and other material degradation.[1] Oxidation reactions can produce free radicals which start chain reactions damaging the host cells.[2] Free radicals are natural by-products of our own metabolism and cause lipid peroxidation in foods which leads to their deterioration.[3] These are electrically charged entities that attack our cells tearing through cellular membranes and react with the nucleic acids, proteins, enzymes etc. present in the body.[2]

It is widely appreciated now that antioxidant activity elicited by the inhibition of generation of free radicals plays a crucial role in providing protection against variety of tissue damages. Several herbs and herbal products are known to possess antioxidant principles and may be useful as organ protective agents.[4,5] Herbs belonging to various families have been reported to possess antioxidant principles like flavonoids and show organ protective properties. In the past few years, there existed an increased preference for antioxidants from natural sources rather than from synthetic sources because of the health risks and toxicity of synthetic antioxidants.

Luffa [*L. cylindrica* (L.) Roem syn *L. aegyptiaca* Mill] commonly called sponge gourd, loofa, vegetable sponge, bath sponge or dish cloth gourd, is a member of cucurbitaceous family. The plant is widely distributed throughout India. Its fruit is used in the traditional medicine as an anthelmintic, carminative, laxative, depurative, emollient, expectorant, diuretic and lactagogue and are also employed in fever, syphilis, tumors, bronchitis, splenopathy and leprosy.[6]

It is applied as a vegetable either prepared like squash or eaten raw like cucumbers.[7,8] Its seeds have been used in the treatment of asthma, sinusitis and fever.[9] The seed oil has been reported to be used for skin infections in the form of tincture.[10,11] The fruit is used in the treatment of ascites, jaundice, and biliary and intestinal colitis and also in enlarged spleen and liver.[12] The plant is reputed

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to have anti tubercular and antiseptic properties.[13,14] The extract of leaves has been used in snake-bites. Although extensive studies have been carried out on fruits and seeds, the pharmacology of the leaves of *L. cylindrica* has remained unexplored as yet. The present investigation was therefore carried out to evaluate *in vitro* antioxidant potential of methanolic extract of *Luffa cylindrica* leaves.

MATERIALS AND METHODS

Plant Material

The leaves of *L. cylindrica* were collected locally from village Pakbara, District Moradabad, Uttar Pradesh, India and were authenticated by Dr. Beena Kumari, Taxonomist, Hindu College, Moradabad (India) as *L. cylindrica* (Cucurbitaceae) leaves. A voucher specimen has been kept in the herbarium (HC.MBD/HAP/BK/2010/5/168) of the Department of Botany, Hindu college, Moradabad (India).

Chemicals and Reagents

All the drugs and chemicals, used in the study, were of analytical grade. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent were obtained from Sigma chemicals USA. Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 2-deoxy-2-ribose and other chemicals used for evaluation of oxidative stress parameters, were obtained from Sisco Research Laboratories, India.

Preparation of Extract

The dried and coarsely powdered plant material (leaves) was extracted with petroleum ether (60-80°) by hot percolation in soxhlet apparatus. The defatted plant material was then extracted with methanol until it became colorless. The extract was concentrated under reduced pressure to yield a crude semi-solid mass. The last traces of the solvent were evaporated under reduced pressure in rotatory evaporator. Standard methods were used for preliminary phytochemical screening of the extract to recognize the phytoconstituents present therein.[15] It was found that the extract contained terpenoids, steroids, flavonoids and glycosides.

Superoxide Scavenging Assay

The scavenging activity of the MELC towards superoxide anion radicals was measured by the method of Liu *et al*.[16] The study was carried out in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system to generate the superoxide anions. It was assayed by the reduction of nitroblue tetrazolium (NBT). For the said purpose 0.75 ml of each of NBT (300 µM) and NADH (936 µM) solutions were mixed with 3 ml of Tris-HCl buffer (100 mM, pH 7.4) to generate the superoxide anion. The mixture was added with 0.3 ml of different concentrations of the extract and standard compound i.e., ascorbic acid. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. Corresponding blanks were taken and % inhibition was calculated. All the tests were performed in triplicate and the graph was plotted with ± SEM of three observations.

Nitric Oxide Scavenging Activity

Griess Illosvoy reaction[17] was used to determine the nitrite oxide ions generated by interaction of oxygen and nitric oxide at physiological pH. Sodium nitroprusside was used as a source of nitric oxide. 10 mM solution of sodium nitroprusside (2 ml) was added in 0.5 ml phosphate buffer saline (pH 7.4) followed by the addition of 0.5 ml of extract of various concentrations. The resulting mixture was incubated at 25°C for 150 min. From the incubated mixture, 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated.[17]

DPPH Assay

The method prescribed by Zeyep *et al*.[18] was used, with minor modifications, for the assay. One ml of 0.1 mM DPPH$^{\bullet}$ in methanol, 450 µl of Tris-HCl buffer and 50 µl aliquot of extract in 50 mM Tris–HCl buffer (pH 7.4) were mixed and incubated in darkness for 30 min. The resultant absorbance was recorded at 517 nm against corresponding blanks (0.01 mM DPPH in methanol). The percentage scavenging was determined and compared with that of ascorbic acid used as the standard.

Scavenging of Hydroxyl Radicals ($^{\bullet}$OH)

The competition between deoxyribose and the extracts for hydroxyl radicals was measured as the determinant for hydroxyl ion scavenging activity. The formed thiobarbituric acid-reactive substances (TBARS)[19] due to attack of the hydroxyl radical on deoxyribose were measured by the method given by Ohkawa *et al*.[20] Briefly, the extracts were added to the reaction mixture containing 2.8 mmol/l deoxyribose, 100 µmol/l $FeCl_3$, 104 µmol/l EDTA, 100 µmol/l ascorbic acid, 1 mmol/l H_2O_2 and 230 mmol/l phosphate buffer (pH 7.4), making a final volume of 1.0 ml. One milliliter of thiobarbituric acid (TBA, 1%) and 1.0 ml trichloroacetic acid (TCA, 2.8%) were added to the test tube and incubated at 100°C for 20 min. After cooling, absorbance was measured at 532 nm against a blank

containing deoxyribose and buffer. Reactions were carried out in triplicate.

Hydrogen Peroxide Radical Scavenging Activity

Hydrogen peroxide scavenging activity of the extract was estimated by method prescribed.[21] A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (pH 7.4). Different concentrations of plant extract and standard ascorbic acid solution viz. 10-100 µg/ml in methanol (1 ml) were added to hydrogen peroxide solution (2 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for back ground subtraction. The experiment was performed in triplicate.

Reducing Power Assay

The Fe^{3+} reducing power of the extract was determined by the method.[22] Extracts of different concentration (10-100 µg/ml) were mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide $[K_3Fe(CN)_6]$ (1%), and then the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 ml of trichloroacetic acid (10%) was added to the mixture which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper layer solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. Ascorbic acid was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the average of three observations. Increased absorbance of the reaction mixture indicated increased reducing power.

Estimation of Total Polyphenol Content

The total polyphenol content (µg/mg extract) was analyzed using the Folin-Ciocalteu reagent method.[23] One hundred milligrams of the MELC extract was dissolved in 250 ml of methanol/water (60:40, *V/V*, 0.3 % HCl) and filtered through a 0.45µm millipore filter. To 100 ml of filtrate, 100 ml of Folin-Ciocalteu reagent (50%, *V/V*) and 2.0 ml of sodium carbonate (2%, *m/V*) were added and mixed completely. After 2 hours, the absorbance of the solution was measured at 750 nm. Quantification was based on the standard curve of gallic acid (0-1.0 mg/ml) dissolved in methanol/water (60:40, *V/V*, 0.3 % HCl). Phenolic content was expressed as milligrams per gram of gallic acid equivalent (GAE).

Statistical Analysis

Results were analyzed statistically using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and expressed as mean ± SE of three observations. Values of $P < 0.05$ were considered significant. The statistical analysis was performed on Graphpad Prism software of version 4. The % inhibition of various radicals was calculated by comparing the results of the test with those of control using the formula.

% inhibition = Abs (Control) - Abs (Test)/Abs (Control) ×100

RESULTS

Phytochemical screening of the crude methanolic extract of the leaves of *Luffa cylindrica* revealed the presence of steroids, flavonoids, terpenoids, glycosides, alkaloids and phenolic compounds. The total phenolic content of the leaf extract was 53.78 ± 1.01 µg/mg of GAE. These phytochemical compounds are known to support biological activities in medicinal plants and thus might be responsible for the antioxidant activities of this plant extract used in this study.

The results of superoxide anion radical scavenging activity of the leaf extract of *Luffa cylindrica* assayed by the PMS-NADH system were as shown in the Table 1. In Figure 1, MELC demonstrated superoxide decomposition activity in a concentration dependent manner with an IC_{50} of 61.02 µg/ml. Higher inhibitory effects of the leaf extracts on superoxide anion formation shown herein possibly rendered them as a promising antioxidant characteristics.

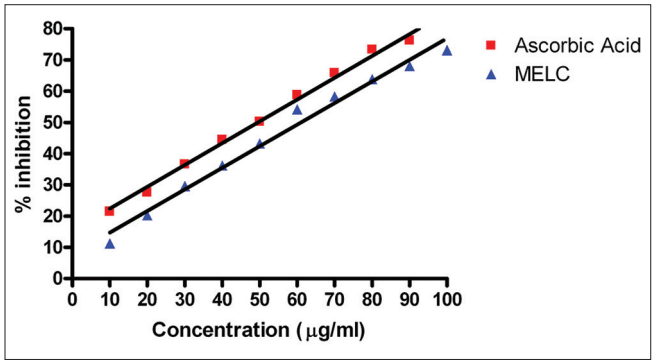

Figure 1: Superoxide scavenging activity of *L. cylindrica* at different concentrations

Table 1: Antioxidant activity of methanolic extract of *L. cylindrica*

Conc (µg/ml)	% inhibition of radicals				
	DPPH	Hydroxyl radical	Hydrogen peroxide	Superoxide	Nitric oxide
10	39.81±0.04	14.28±0.06	10.99±0.07	11.31±0.14	8.573±0.18
20	46.29±0.05	21.42±0.13	12.88±0.05	20.32±0.19	13.74±0.19
30	52.77±0.07	25.00±0.11	16.32±0.08	29.6±0.26	18.4±0.19
40	57.4±0.07	31.42±0.17	20.83±0.09	36.2±0.19	29.24±0.27
50	62.96±0.06	35.71±0.09	23.68±0.08	43.32±0.12	34.19±0.19
60	68.51±0.11	39.28±0.13	30.97±0.07	54.23±0.17	43.94±0.22
70	70.37±0.09	53.57±0.07	36.61±0.05	58.3±0.14	54.32±0.20
80	74.67±0.08	57.14±0.08	40.03±0.05	63.82±0.14	59.33±0.19
90	79.62±0.09	60.08±0.11	48.92±0.03	68.09±0.21	65.75±0.22
100	85.18±0.12	63.18±0.07	52.63±0.10	73.17±0.18	68.03±0.21

Data presented as ±SEM of each triplicate test. $P<0.05$ was considered significant

The results suggested that the plant extract had a potent superoxide radical scavenging effect.

Nitric oxide inhibitors have been shown to have beneficial effects on some aspect of inflammation and tissue damage seen in inflammatory diseases. MELC significantly inhibited [Figure 2] nitric oxide in a dose dependent manner [Table 1] with the IC_{50} being 69.49 µg/ml. The results indicated that the extract might contain compounds capable of inhibiting nitric oxide and offered scientific evidence for the use of the leaves in inflammatory conditions.

The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It was visually noticeable by a color change from purple to yellow. The methanolic extract of *L. cylindrica* and ascorbic acid showed DPPH radical scavenging activity in a concentration–dependent manner [Figure 3]. MELC exhibited maximum scavenging activity of 85.18% whereas that of the ascorbic acid was 76.61 %. The IC_{50} values were as shown in Table 2.

Activity of the different concentrations of MELC on hydroxyl radical has been shown in Table 1. MELC exhibited concentration dependent scavenging activity against hydroxyl radical generated [Figure 4]. The IC_{50} value of extract was found to be 72.33 µg/ml. The observed dose dependent scavenging effect could be explained by understanding the nature and generation of radicals as well as studying different physical and chemical properties of the naturally occurring antioxidant.

As shown in Figure 5, MELC also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC_{50} of 97.50 µg/ml. The decomposition of H_2O_2 by MELC might have resulted from its antioxidant and free radical scavenging activity.

Table 1 showed the reducing power of the *Luffa cylindrica* methanolic extracts as a function of their concentration. In this assay, the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of each compound. Presence of reducers caused the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. By measuring the formation of Perl's Prussian blue at 700 nm, it was possible to determine

Table 2: IC_{50} of ascorbic acid and methanolic extract of *L. cylindrica*

Activity	IC_{50} (µg/ml)	
	MELC	Ascorbic acid
DPPH	26.46	38.45
Hydroxyl radical	72.33	50.83
H_2O_2 scavenging	97.50	87.88
Superoxide scavenging	61.02	49.55
Nitric oxide scavenging	69.49	57.64

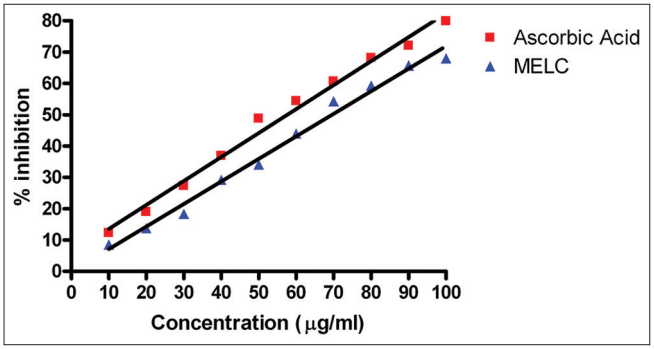

Figure 2: Nitric oxide scavenging activity of *L. cylindrica* at different concentrations

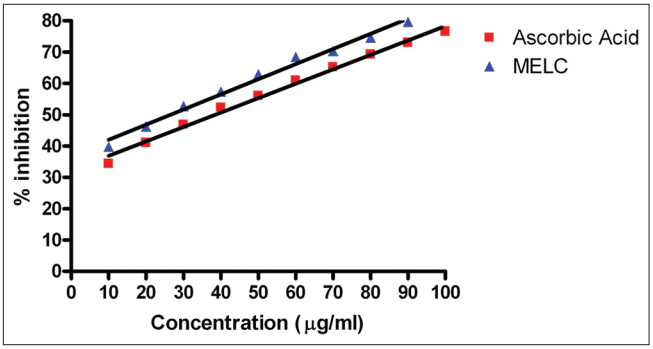

Figure 3: DPPH scavenging activity of *L. cylindrica* at different concentrations

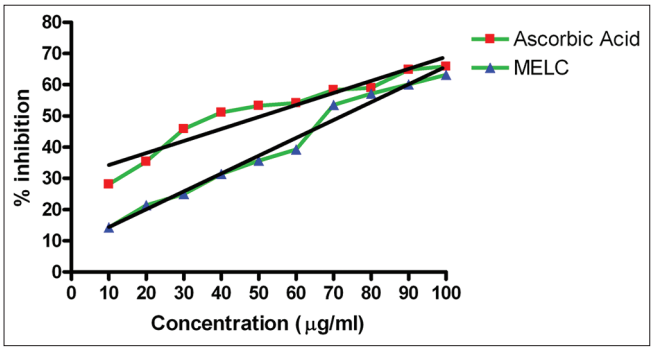

Figure 4: Hydroxyl radical scavenging activity of *L. cylindrica* at different concentrations

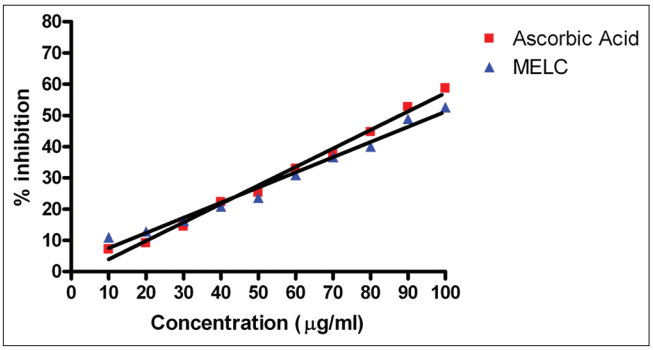

Figure 5: Hydrogen peroxide scavenging activity of *L. cylindrica* at different concentrations

the Fe^{2+} concentration. The reducing power of the MELC increased with their concentrations [Figure 6]. The result revealed that MELC did consist of polyphenolic compounds inducing the greater reducing power.

DISCUSSION

In the present investigation potent antioxidant activity of *L. cylindrica* leaf extract was observed using different methods. However the efficacy of extract to scavenge the different radicals differed in each method depending upon the involved mechanism of free radical scavenging and adopted assay methodology.

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers. Its univalent function reduces molecular oxygen. It can also reduce certain iron complexes such as cytochrome.[24] The present study showed potent superoxide radical scavenging activity of *L. cylindrica* leaf extract. The scavenging activity of such radical by the plant extract when compared favorably with the standard reagents such as ascorbic acid suggested that the plant might also serve as a potent scavenger of superoxide radical.

Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell.[25] Scavenging of H_2O_2 by the plant extracts may be attributed to their phenolic contents, which donate electron to H_2O_2, thus reducing it to water. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner.

Nitric oxide is a free radical product in mammalian cells involved in the regulation of various physiological processes. However, excess production of nitric oxide radical is associated with several diseases.[26] In the present study the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25°C was reduced by the methanolic extract of *L. cylindrica*. This might be due to the antioxidant principles present in the extract which competed with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite.

The result of DPPH scavenging activity assay in this study indicated that the plant was potently active. This suggested that the plant extract contained compounds that were capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. The scavenging activity of DPPH radical by the plant extract was found to be appreciable; this implied that the plant extract might be useful for treating radical related pathological damages especially at higher concentration.[27]

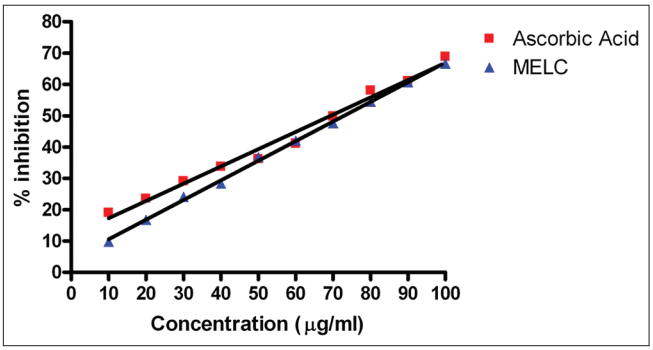

Figure 6: Percentage reducing ability of *L. cylindrica* at different concentrations

Hydroxyl radical is highly reactive oxygen centered radical formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes and most biological molecules it contacts[28] and is known to be capable of abstracting hydrogen atoms from membrane lipids[29] that brings about peroxidation reaction of lipids. In the present study a significant correlation existed between the concentration and hydroxyl radical scavenging ability of extract.

CONCLUSION

The results obtained in the present investigation indicated that the methanolic extract of leaves of *L. cylindrica* exhibited free radical scavenging activity. The overall antioxidant activity of the leaves extract might be attributed to the presence of secondary metabolites. The findings of the present study suggested that *L. cylindrica* leaves could be a potential source of natural antioxidant having great importance as therapeutic agent in preventing or slowing the oxidative stress related degenerative diseases.

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