

IN VITRO ANTIOXIDANT ACTIVITY OF *LAGENARIA SICERARIA* LEAVES

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The free radical scavenging potential of methanolic extract of Lagenaria siceraria leaves (MELS) was studied on in vitro antioxidant models. The antioxidant potential was evaluated by determining the activity of hydroxyl and hydrogen peroxide (H₂O₂) radicals scavenging and 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay. In all these studies, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals. The extract was also shown to have high phenolic content i.e. 99.09±0.10 µg/mg. These results clearly indicated that MELS could be a potential source of natural antioxidant and effective against free radical mediated diseases.

Keywords: Antioxidant, In vitro, *Lagenaria siceraria*, Reactive oxygen species

INTRODUCTION

Free radicals, often called reactive oxygen species (ROS), are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Tiwari 2001). They are generated as by-products of biological reactions or from exogenous factors. When ROS production is greater than the detoxification capacity of the cell, excessively generated ROS causes extensive damage to DNA, proteins, lipids etc. and acts as a mediator of pro-inflammatory and carcinogenic events (Kowaltowski and Vercesi 1999). Such conditions are considered to be important causative factors in the development of diseases such as diabetes, stroke, arteriosclerosis, cancer and cardiovascular diseases (Yamaguchi *et al.* 2000). In the treatment of such diseases, antioxidant therapy has gained an immense importance. These antioxidants interfere with the oxidative processes by reacting with free radicals, chelating catalytic metal ions and also by acting as oxygen scavengers (Buyukokuroglu, Oktay and Kufrevioglu 2001). The biological effects of dietary antioxidants have generated a lot of interests in the modern era due to their potent antioxidant activities, absence of side effects and economic viability (Auudy *et al.* 2003). Many scientists have tried to obtain dietary antioxidants such as ascorbate, tocopherol and carotenoids from fruits and vegetables as they could help in protecting cells from cellular damages induced by oxidative stress.

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The plant, *Lagenaria siceraria* (Molina) standl. (Family: Cucurbitaceae), known as bottle gourd, is a common fruit vegetable used throughout India. Since time immemorial the fruit has been used as immunosuppressant (Sankari *et al.* 2010), diuretic (Ghule *et al.* 2007), cardio-tonic, cardio-protective (Hassanpour, Bodhankar and Dikshit 2008) and nutritive agent (Rahman *et al.* 2008). The fruit has been also reported to possess good source of vitamin B complex and choline along with fair source of vitamin C and β -carotene (Kirtikar and Basu 2001). It is also reported to contain cucurbitacins, fibres and polyphenol (Nadkarni 1954). Among other activities reported with the fruits of *L. siceraria* include antioxidant activity (Shirwaikar and Sreenivasan 1996) and hypolipidemic in triton-induced hyperlipidemic rats (Jiwajinda *et al.* 2002). HPLC analysis of methanol extract from plant showed the presence of flavones-c glycosides. Lagenin (a ribosome inactivating protein) isolated from the seeds of *L. siceraria* possessed immuno-protective, antitumor, anti HIV and antiproliferative properties (Wang and Ng 2000). Aerial parts of the plant also have been reported to have antihyperglycemic (Saha *et al.* 2011a) and anticancer (Saha *et al.* 2011b) activities.

In view of the immense medicinal importance of the plant, the present investigator focused onto exploration of free radical scavenging activity of the leaves of this plant to determine and establish its role in various oxidative stress conditions generated by various reactive oxygen species.

METHODS

Chemicals and Reagents

All the drugs and chemicals used in the study were of analytical grade. 1,1-diphenyl-2-picryl hydrazyl (DPPH) and Folin-Ciocalteu reagent were obtained from Sigma Chemicals (St. Louis, MO, 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 (Mumbai).

Plant Material

The leaves of *L. siceraria* were procured locally from Pakbara village, Moradabad District of Uttar Pradesh, India and were identified by Dr. Beena Kumari (taxonomist, Hindu College, Moradabad, India) as *L. siceraria* (Molina) standl. (Cucurbitaceae) leaves. A voucher specimen was preserved at the herbarium (HC.MBD/HAP/BK/2010/7/167) in the Department of Botany, Hindu College, Moradabad for further references. Leaves were washed with tap water, dried in shade and were then ground to coarse powder and stored in an airtight container.

Preparation of Extract

The dried and coarsely powdered plant material 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 colourless. 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 recognise the phytoconstituents present in the extract (Harborne 1984). It was concluded that the extract contained terpenoids, steroids, flavonoids and tannins.

In Vitro Antioxidant Activity

DPPH assay

The ability of the extracts to scavenge DPPH radicals (DPPH[•]) was determined according to the method prescribed (Zeyep, Muberra and Esra 2007) with minor modifications. A 50 µL aliquot of extract, in 50 mM Tris-HCl buffer (pH 7.4), was mixed with 450 µL of Tris-HCl buffer and 1.0 mL of 0.1 mM DPPH[•] in methanol. After 30 min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm against corresponding blanks (0.01 mM DPPH in methanol) and ascorbic acid was used as standard. All the tests were performed in triplicate and the graph was plotted with ±SEM of three observations.

Superoxide Scavenging Assay

The previously described (Liu and Ng 2000) method was used to investigate the superoxide anion radical scavenging activity of the methanolic extract of *L. siceraria* leaves (MELS). For the said purpose a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used to generate the superoxide anion through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of NBT. To generate the superoxide anion, 3 mL of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 mL of NBT (300 µM) solution, 0.75 mL of NADH (936 µM) solution and 0.3 mL of different concentrations of the extract were used. 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 on spectrophotometer.

Scavenging of Hydroxyl Radicals

The competition for hydroxyl radicals generated from the Fe³⁺/ascorbate/EDTA/hydrogen peroxide (H₂O₂) system, between deoxyribose and the extracts, was measured in the study. These hydroxyl radicals attack on deoxyribose lead to the formation of thiobarbituric acid-reactive substances (TBARS) (Halliwell and Gutteridge 1981). The formed TBARS were measured by a previously described method (Ohkawa, Ohishi and Yagi 1979). The extracts were added to the reaction mixture containing 2.8 mmol/L deoxyribose, 100 µmol/L FeCl₃, 104 µmol/L EDTA, 100 µmol/L ascorbic acid, 1 mmol/L H₂O₂ and 230 mmol/L phosphate buffer (pH 7.4), making a final volume of 1.0 mL. One mL of TBA (1%) and 1.0 mL 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. The percentage inhibition of hydroxyl radical by the extract was determined by comparing the absorbance values of the control and experimental tubes.

Hydrogen Peroxide (H₂O₂) Radical Scavenging Activity

H₂O₂ scavenging activity of the extract was estimated by a previously prescribed method (Sroks and Cisowski 2003). A solution of H₂O₂ (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 H₂O₂ solution (2 mL). Absorbance of H₂O₂ at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H₂O₂. For each concentration, a separate blank sample was used for back ground subtraction. The experiment was performed in triplicate.

Nitric Oxide Scavenging Activity

Nitric oxide radical scavenging activity was determined according to the reported method (Garraat 1964). SNP in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be determined by the use of the Griess Illosvoy reaction. Two mL of 10 mM SNP in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract at various concentrations and the 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 naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm on the spectrophotometer. Consequently nitric oxide radical scavenging activity was calculated.

Estimation of Total Polyphenol Content

The total polyphenol content (µg/mg extract) was analysed using the Folin-Ciocalteu reagent method (Singleton, Orthofer and Ramuela-Raventos 1999). One hundred mg of the MELS extract was dissolved in 250 mL of methanol/water (60:40, V/V, 0.3% HCl) and filtered through a 0.45 µm 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 hrs, 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).

Reducing Power Assay

The Fe³⁺ reducing power of the extract was determined by previously described method (Oyaizu 1996). The methanolic extract (10–100 µg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [K₃Fe(CN)₆] (1%), and then the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 mL of TCA acid (10%) was added to the mixture and then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of upper layer of the solution was mixed with 2.5 mL distilled water and 0.5 mL FeCl₃ (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.

Statistical Analysis

The results are expressed as mean \pm standard error (mean \pm SE) of three observations. The % inhibition of various radicals was calculated by comparing the results of the test with those of control using the formula (Shirwaikar, Rajendran and Dinesh Kumar 2004).

$$\% \text{ inhibition} = \frac{\text{absorbance (control)} - \text{absorbance (test)}}{\text{absorbance (control)}} \times 100$$

RESULTS

Several concentrations ranging from 10–100 $\mu\text{g/mL}$ of the methanolic extract were compared for their antioxidant activity in different in vitro models. It was observed that free radicals were scavenged by the extracts in a concentration dependent manner (within the predetermined concentration range) in all the models.

DPPH Radical Scavenging Activity

Free radicals scavenging activity of DPPH has been widely used to evaluate the antioxidant activity of natural products obtained from plant and microbial sources. In DPPH scavenging activity model it was observed that MELS (10–100 $\mu\text{g/mL}$) significantly scavenged DPPH \cdot in a concentration dependent manner. However extract showed weak scavenging activity in lower concentrations; the higher concentrations (50–100 $\mu\text{g/mL}$) exhibited promising DPPH \cdot scavenging activity ranging from 36.26% to 65.93% (Table 1). DPPH is a relatively stable free radical and the assay determines the ability of MELS to reduce DPPH \cdot to the corresponding hydrazine by converting the unpaired electrons to form pairs. This conversion is the action of the antioxidant.

Superoxide Radical Scavenging Activity

The superoxide anion radical scavenging activity of *L. siceraria* leaves extract assayed by the PMS-NADH system is shown in Table 1. The superoxide scavenging activity of MELS was increased markedly with the increase in concentrations of the extract. Thus, higher inhibitory effects of the extract on superoxide anion formation noted herein possibly rendered its promising antioxidant potential. The half inhibition concentration (IC_{50}) of MELS was 63.49 $\mu\text{g/mL}$ (Table 2). These results suggested that MELS had a potent superoxide radical scavenging effects.

Hydroxyl Radical Scavenging Activity

Activity of the different concentrations of MELS on hydroxyl radical had been as shown in Table 1. MELS exhibited concentration dependent scavenging activity against generated hydroxyl radical. The IC_{50} value of extract was found to be 56.15 $\mu\text{g/mL}$ (Table 2). 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.

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

Conc ($\mu\text{g/mL}$)	% inhibition of radicals				
	DPPH	Hydroxyl radical	H ₂ O ₂	Superoxide	Nitric oxide
10	9.34 \pm 0.78	26.08 \pm 1.65	7.70 \pm 0.93	12.53 \pm 1.59	9.43 \pm 0.13
20	15.93 \pm 0.42	34.78 \pm 1.13	8.40 \pm 0.65	22.18 \pm 1.14	15.39 \pm 0.60
30	29.67 \pm 1.61	43.47 \pm 0.62	10.13 \pm 1.32	31.25 \pm 2.18	20.67 \pm 0.62
40	31.31 \pm 0.91	47.82 \pm 1.87	12.76 \pm 0.75	36.27 \pm 2.22	28.12 \pm 0.39
50	36.26 \pm 1.18	52.17 \pm 0.94	13.27 \pm 0.24	43.18 \pm 2.58	37.51 \pm 2.13
60	41.20 \pm 2.29	53.14 \pm 1.74	16.71 \pm 1.13	48.68 \pm 2.38	41.90 \pm 0.65
70	47.25 \pm 2.45	56.52 \pm 2.05	19.25 \pm 1.67	52.41 \pm 2.11	49.94 \pm 0.90
80	52.19 \pm 1.63	57.35 \pm 1.04	21.88 \pm 1.98	59.62 \pm 3.75	58.72 \pm 2.04
90	58.79 \pm 2.27	60.86 \pm 2.46	25.43 \pm 1.70	67.28 \pm 2.69	64.24 \pm 1.99
100	65.93 \pm 3.13	65.21 \pm 1.57	31.81 \pm 0.47	72.46 \pm 3.01	66.13 \pm 2.05

Note: Data presented as \pm Standard Error Mean (SEM) of each triplicate test. $p < 0.05$ was considered significant

Table 2: Free radical scavenging ability of *L. siceraria* and ascorbic acid.

Activity	IC ₅₀ ($\mu\text{g/mL}$)	
	MELS	Ascorbic acid
DPPH	73.98	38.49
Hydroxyl radical	56.15	50.91
H ₂ O ₂ scavenging	186.37	60.29
Superoxide scavenging	63.49	49.57
Nitric oxide scavenging	71.00	57.64

Hydrogen Peroxide Radical Scavenging Activity

MELS also demonstrated H₂O₂ decomposition activity in a concentration dependent manner with an IC₅₀ of 186.37 $\mu\text{g/mL}$ (Table 2). The decomposition of H₂O₂ by MELS might have partly resulted from its antioxidant and free radical scavenging activity.

Nitric Oxide Scavenging Activity

MELS significantly inhibited nitric oxide in a dose dependent manner (Table 1) with the IC₅₀ being 71.00 $\mu\text{g/mL}$. The result indicated that the extract might contain compounds able to inhibit nitric oxide and offered scientific evidence for the use of the leaves in oxidative stress conditions.

Reducing Power Activity

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Similar to the antioxidant activity, the reducing power of MELS increased with increasing concentration. The result showed that MELS consists of hydrophilic poly phenolic compounds that might have caused the greater reducing power.

Amount of Total Phenolic Compounds

In the present study, total phenolic content present in extract was estimated using modified Folin-Ciocalteu method. The extract was found to contain 99.09 ± 0.10 $\mu\text{g}/\text{mg}$ total polyphenolics expressed as GAE (micrograms per milligram of GAE).

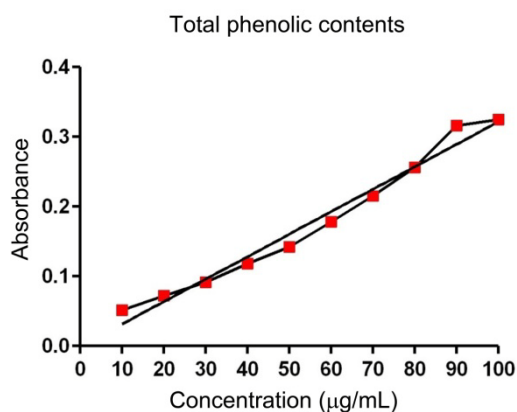


Fig. 1: Gallic acid standard graph for total phenolic contents of *L. siceraria*.

DISCUSSION

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radical can bring about many adverse reactions leading to extensive tissue damage. Lipid proteins are all susceptible to attack by free radical. Many plant species with antioxidant activities act as protective agents against these radicals. In the present investigation potent antioxidant activity of *L. siceraria* leaf extract was observed using different methods. However the efficacy of extract to scavenge the different radicals differed in each method depending upon the mechanism of free radical scavenging and assay methodology.

The result of DPPH scavenging activity assay in this study indicated that the plant was potentially active. This suggested that the plant extract did contain compounds that could be capable of donating hydrogen to a free radical in order to remove the odd electron which is responsible for the radical's reactivity.

Hydroxyl radical is highly reactive oxygen centred 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

(Aruoma 1999) and is known to be capable of abstracting hydrogen atoms from membrane lipids (Yen and Duh 1994) and brings about peroxidic reaction of lipids. In the present study a significant correlation existed between the concentration and hydroxyl radical scavenging ability of the extract.

H₂O₂ is a weak oxidising agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly. Once inside the cell, H₂O₂ can probably react with Fe²⁺, and /or Cu²⁺ ions to form hydroxyl radical and this might be the origin of many of its toxic effects (Halliwell and Gutteridge 1981). It is therefore biologically advantageous for cells to control the amount of H₂O₂ getting accumulated. Scavenging of H₂O₂ by the plant extract could be attributed to its phenolics which donate electron to H₂O₂, thus reducing it to water. The extract was capable of scavenging H₂O₂ in a concentration dependent manner.

It is well known that superoxide anions damage biomolecules directly or indirectly by forming H₂O₂, •OH, peroxy nitrite or singlet oxygen during aging leading to pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation (Yen and Duh 1994). The scavenging activity of this radical by the plant extract compared favourably with the standard reagent suggesting that the plant could also be a potent scavenger of superoxide radical. The probable mechanism of superoxide scavenging would be attributed to the inhibitory effects of MELS towards generation of superoxide in the in vitro reaction system.

Nitric oxide is an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, control of vasodilation and control of blood pressure (Rees, Palmer and Moncada 1989; Palmer, Ferrige and Moncada 1987) etc. However, the elevation of the NO• results in several pathological conditions including cancer. Moreover in the pathological conditions, nitric oxide reacts with superoxide anion and form potentially cytotoxic molecules, peroxynitrite. Nitric oxide inhibitors have been shown to have beneficial effects on some aspects of inflammation and tissue damage seen in inflammatory diseases. The level of nitric oxide was significantly reduced in this study by the crude extract. Since NO• plays a crucial role in the pathogenesis of inflammation, this may explain the use of *L. siceraria* for the treatment of inflammation.

For the measurements of the reducing ability, the Fe³⁺-Fe²⁺ transformation was investigated in the presence of MELS. Such reducing capacity of a compound might serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants would have been assigned to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Yildirim *et al.* 2000).

Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al.* 1995). The preliminary photochemical analysis suggested the presence of terpenoids, steroids, flavonoids and tannins in the methanolic extract; hence the observed antioxidant activity could be due to the presence of any of these constituents in the extract. However, the confirmation of activity by these constituents requires further analysis and would be the matter of investigation in our group.

CONCLUSION

The results obtained in the present study indicated that *L. siceraria* leaves extract exhibited free radical scavenging activity against hydroxyl, peroxide and DPPH[•]. The overall antioxidant activity of MELS might be attributed to its polyphenolic content and other phytochemical constituents. The findings of the present study suggested that *L. siceraria* leaves could be a potential source of natural antioxidant that would have great importance as therapeutic agents in preventing or slowing the progress of reactive oxygen species and associated oxidative stress related degenerative diseases.

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