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INVITRO STUDY ON PHYTOCHEMICAL SCREENING AND ANTIOXIDANT PROPERTIES FROM ETHYL ACETATE EXTRACT OF *LAGENARIA SICERARIA*.

[1]DR. K. Rajeswari and [2]DR. T. Sasipriya [3]DR V. Ramadass[4]DR.S.Sankaranarayanan

[1] Emergency Medical officer, National Institute of Siddha, Sanatorium, Chennai, 48, Tamilnadu, India
[2]Cure Siddha Healthcare Centre, Thirunelveli, Tamilnadu, India
[3]Post graduate Scholar, Department of Puramaruthuvam, Government Siddha Medical College, Arumbakkam, Chennai- 600 106, Tamilnadu, India
[4]Assitant Professor, Department of Medicinal Botany, Goverment Siddha Medical College,arumbakkam, Chennai, tamilnadu, india
Email: rajikarvannan@gmail.com; ramdassbsms3@gmail.com
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ABSTRACT

Lagenaria siceraria is a rich source of bioactive compounds. The goal of this study was to test different extracts viz ethyl acetate of selected plant their phenolic contents, and antioxidant potential. Ethyl acetate, extract was used to extract bioactive phytochemicals from *L. siceraria*. Recovered extracts were studied in terms of total phenolic compounds, total flavonoids and antioxidant properties. Ethyl acetate is the best solvents for extracting phenolics (ca. 8.6 g GAE g^{-1} extract) and flavonoids (12.7 mg QE g^{-1} extract). Antioxidant potential of *L. siceraria* extracts were estimated using ABTS"+ 2, 2 azino bis-(3- ethylbenzthiazoline-6-sulfonic acid) radical scavenging assay, Inhibition of lipid peroxidation, Super oxide radical scavenging activity, Nitric oxide radical scavenging activity. ABTS"+ scavenging activity of *L. siceraria* 72.6%. *L. siceraria* ethyl acetate extract inhibited lipid peroxidation range of 67.32%. Superoxide radicals, metal chelating and nitric oxide inhibition also showed highest antioxidant in ethyl acetate extract of *L. siceraria*. In conclusions using ethyl acetate extract of *L. siceraria* in food or pharmaceutical products may be an effective antioxidant strategy. Data from this study might be used for developing natural preservatives and bioactive agents with health promoting activities.

Key words: *Lagenaria siceraria*

INTRODUCTION

Fruits and vegetables are reflected to be the major contributors of Reactive Oxygen Species (ROS)-scavenging antioxidants. The cancer and other disease preventing action probably resides in the fact that vegetables contain not only abundant nutritional antioxidants, but also a great quantity of non-nutritional antioxidants, such as flavonoids (quercetin, one of the most abundant flavonoids present in vegetables (Wach et al., 2007), betalains, S-allyl cysteine, and S-methyl cysteine (Murcia et al., 2006). Flavonoids have numerous beneficial effects on human health, acting as antioxidants because of their ability to act against a wide range of cations in multiple hydroxyl groups and in the carbonyl group on ring C (Shaghaghiet al., 2008). Lifestyles that involve diets high in vegetables and fruits have been associated with a reduced risk of cancer and this association has motivated the “5-a-day” program. Furthermore, vegetables are very low in calories and are usually consumed in their fresh state, and also after processing and cooking. A calorie-restricted diet decreases chemically induced tumor incidence and

increases life expectancy, reducing oxidative damage, and altering rates of cell division and/or apoptosis. This fact not only attenuates the generation of ROS by liver mitochondria, but also alters the activities of the electron transport chain (Hwang and Bowen, 2007). However, cooking, such as boiling, which causes overall flavonol losses (Makris and Rossiter, 2001), microwaving (Zhan and Hamauzu, 2004), pressure-cooking, griddling, baking, and frying (Young and Jolly, 1990), can profoundly affect both the texture and the nutritional value of vegetables. Cooking softens the cell walls and facilities the extraction of carotenoids (Rodrıguez-Amaya, 1999). Some studies have shown that a loss of vitamins in vegetables during cooking varies with the cooking treatment (Lin and Chang, 2005).

Vegetables contain several hydrophilic and lipophilic antioxidant compounds and it is important to estimate the antioxidant activity using different methods. They may act together more effectively than singly because they function synergistically and are capable of quenching free radicals in both aqueous and lipid phases (Ohr et al., 2004). Antioxidant compounds may also act as metal chelators and interfere with the pathways that regulate cell division and proliferation and detoxification; they also may regulate inflammatory and immune responses, and may have anti-ulcerative properties (Hamauzuet al., 2008). They may inhibit or activate a large variety of mammalian enzyme systems, exhibiting biphasic dose responses in cells at low doses. Phytochemicals activate signaling pathways that result in the increased expression of genes-encoding cytoprotective proteins, including antioxidant enzymes (Dragstedet al., 2006), protein growth factors, and mitochondrial proteins. Examples of the phytochemicals: sulphorane or isothiocyanates (present in broccoli) and allicine (present in garlic) (Mattson, 2008).

Several techniques have been used to determine the *in vitro* antioxidant activity in order to allow rapid screening of promising substances and/or mixtures. In the last few years, evaluation of the antioxidant potential of food, as well as natural, pharmaceutical, and cosmetic products has been increasing. The interest on this field began to expand in the 1990s based on the observation that many natural products have beneficial effects on human health. Many methods are available for analyzing antioxidant activity, with different concepts, mechanisms of action, ways of expressing results, and applications (Huang et al., 2005).

On the one hand, indirect methods, involving electron transfer reactions, such as ABTS•+, DPPH•, and FRAP, are easier to apply, but present some limitations. In this case, the methods evaluate the free radical scavenging ability of antioxidant compounds, and this does not necessarily correspond to the real oxidative degradation, although, in some circumstances, the donation of hydrogen atoms (or electrons) correlates with the antioxidant activity. Therefore, since these chemically distinct methods are based on different reaction mechanisms, it is important to use different methods in order to obtain a more thorough assessment of the antioxidant potential of a sample (Ou et al., 2002).Several analytical methods have been used to evaluate antioxidant activity by free radical scavenging, but besides being one of the fastest, ABTS•+ method also provides good solubility, which allows the analyses of both lipophilic and hydrophilic compounds (Re et al., 1999). *Lagenaria siceraria* (Molina) standley (LS) (Family: Cucurbitaceae) is an annual herbaceous climbing plant with a long history of traditional medicinal uses in many countries, especially in tropical and subtropical regions. *Stems* are prostrate or climbing, angular, ribbed, thick, brittle, softly hairy, upto 5 m long, cut stems exude no sap. The fruits, leaves, oil, and seeds are edible and used by local people as folk medicines in the treatment of jaundice, diabetes, ulcer, piles, colitis, insanity, hypertension, congestive cardiac failure, and skin diseases. The fruit pulp is used as an emetic, sedative, purgative, cooling, diuretic, anti-bilious, and pectoral. The flowers are an antidote to poison.

MATERIALS AND METHODS

PLANT MATERIALS

Fruit of *Lagenaria siceraria* were purchased from K. K. Nagar vegetable market Chennai 600 078, TamilNadu, India. Plant was authenticated by Dr. S. Sankaranarayanan, Head, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai-600 106, Tamilnadu.

PREPARATION OF EXTRACTS

Organic solvents (Aqueous and Ethyl acetate) extract from the fruit of *L. siceraria* were prepared according to the method described by Boaky- Yiadon (1979). Hundred grams of fruit of *L. siceraria* were blended into paste using an electric blender for each solvent. The blended material was transferred to a beaker and mixed separately in 100 ml of the organic solvent at room temperature. The mixture was extracted by agitation on a rotary shaker. The extract obtained was vaccum-dried and used for further test.

PHYTOCHEMICAL SCREENING

The aqueous extract of fruit of *L. siceraria* were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Harborne 1973; Trease and Evans 1983).

TOTAL PHENOLIC CONTENT

The total phenolic content (TPC) of aqueous extracts fruit of *L. siceraria* was determined using the method by Gutfinger (1981). The methanol extract (1 mL, 1 mg/mL) was mixed thoroughly with 1 mL of 50% Folin-Ciocalteu reagent and 1 mL of 2% Na_2CO_3, and centrifuged at 13400X *g* for 5 min. The absorbance of upper phase was measured using a spectrophotometer (ELICO (SL150) UV–Vis Spectrophotometer) at 750 nm after 30 min incubation at room temperature. Total phenolic content was expressed as a catechol equivalent.

ESTIMATION OF FLAVANOID

A 1ml aliquot of each aqueous extract of fruit of *L. siceraria* was mixed thoroughly with 1ml of 2% aluminium chloride and 0.5 ml 0f 33% acetic acid followed by the addition of 90% methanol and the content is thoroughly stirred and allowed to stand for 30 minutes (Delcour and de Varebeke, 1985). The absorbance was measured at 414 nm using a UV-Visible Spectrophotometer. Quercetin was used as a standard.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography of ethyl acetate extract of fruit of *L. siceraria* was performed using standard procedures (Harborne 1973) .The aqueous methanol extract was placed carefully in precoated aluminum silica gel 60 F, Merck F 254 using a microcapillary tube. The spots were allowed to dry for few minutes and the TLC plate was placed in the solvent mixture, Toluene, acetone and Formic acid (6:6:1). After drying, the TLC plates were observed under UV at 240nm and 360 nm in UV TLC viewer.

ABTS RADICAL SCAVENGING ASSAY

ABTS radical scavenging activity of ethyl acetate extract of composed leaves of fruit of *L. siceraria* was followed by Re et al. (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734nm against

distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.
ABTS Scavenging Effect (%) = [(A_0- A_1/A_0) ×100] Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of extract.

INHIBITION OF LIPID PEROXIDATION ACTIVITY

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer and different concentrations of ethyl acetate extract of fruit of *L. siceraria* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.
Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

SUPEROXIDE RADICAL SCAVENGING ASSAY

This assay was based on the capacity of the ethyl acetate extract of fruit of *L. siceraria*to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution:
% Super oxide radical scavenging capacity= [(A_0-A_1)/A_0] ×100
Where A_0 was the absorbance of control and A_1 was the absorbance of both plant extracts fraction.

NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

Nitric oxide scavenging ability of ethyl acetate extract fruit of *L. siceraria* was measured according to the method described by Olabinri et al. (2010). 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of extracts and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard.

RESULT AND DISCUSSION
PHYTOCHEMICAL SCREENING

The phytochemical screening of the fruit of aqueous extract of *L. siceraria* studied presently showed the presence of alkaloids, flavonoids, polyphenol, terpenoids, and absence of glycosides and tannin.

S/No.	Constituents	Aqueous extract of *L. siceraria*
1.	**Alkaloids-Dragendroffs reagent**	+
2.	**Flavonoids- Alkalaine**	+
3.	**Tannin- Fecl$_3$ test**	-
4.	**Saponins- Frothing test**	+
5.	**Terpenoids - Nollers test**	+
6.	**Glycosides- Keller-Killiani Test**	-
7.	**Polyphenols-Ferrozine**	+
8.	**Anthocynin- Ammonia Test**	+

+ Indicate Positive; -Indicate negative

PARTIAL CHARACTERIZATION OF ETHYL ACETATE EXTRACT FRUIT OF *L. SICERARIA*BY TLC

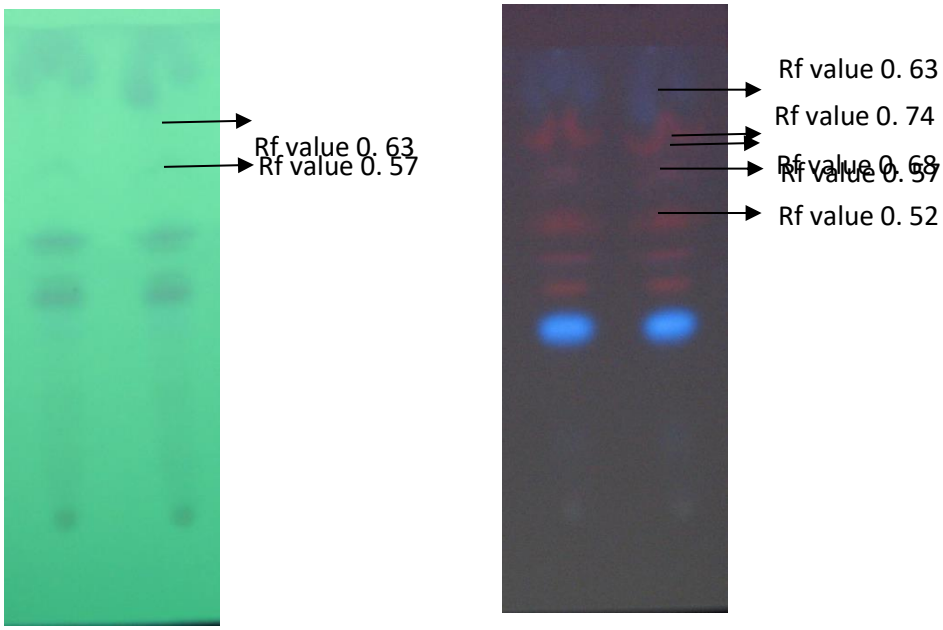
Partial characterization of the ethyl acetate extract of fruit of *L. siceraria* were adsorbed on precoated siliga gel TLC plates (60 F_{254}, Merk, USA). Efficient solvent system used Toluene: Acetone: Formic acid in the ratio 6:6:1 were used for the mobile phase of phytochemical and followed by colour development of the separated compounds phytochemical was viewed under UV 240 nm and 360 nm (Table-2).

Table-2. Partial characterization of ethyl acetate extract of fruit of *L. siceraria*by TLC

Component No.	Ethyl acetate extract of *L. siceraria* Fruit	
	UV Light 360nm Rf value	**UV light 240 nm Rf value**

1.	0.74	-
2.	0.68	-
3.	0.63	063
4.	0.57	0.57
5.	0.52	-

Fig-1. Partial characterization of ethyl acetate extract of fruit of *L. sicerariaby* TLC



TOTAL PHENOLIC AND FLAVONOID CONTENT FRUIT OF *L. SICERARIA*

The total phenol yield of fruit of *L. siceraria* fresh extracts recovered from ethyl acetate extract under study was 10.7 g extract/100 g and also in ethyl acetate extract yield phenolic content 7.3 g extract/100 g. Total phenol and flavonoid content of ethyl acetate extract the highest amount (12.7 mg extract/100 g equivalent to Gallic acid and 60.3 mg extract/100 g equivalent to rutin). Plant phenolics have shown to possess antioxidant, hypocholesterolemic, hypolipidimic, anti-hypertensive, anti-diabetic and anti-cancerous properties. Cucurbitaceae fruits to health improvement has generally been partly

associated with their antioxidant capacity, being phenolic compounds the major antioxidants of these plants (Podsedek 2007; Jahangir et al., 2009).

FREE RADICAL-SCAVENGING ABILITY USING ABTS ASSAY OF FRUIT OF *L. SICERARIA*

The radical scavenging ability was measured by ABTS assay as per given in table 3. The inhibition percentage of the ABTS radical activity was assessed on average and high free radical-scavenging values were found in ethyl acetate extract of *L. siceraria.* In ABTS assay, inhibition percentage of the pure ascorbic acid was lower than *L. siceraria* (Table-3). Nevertheless, in present study, it is showed that these activities were mainly due to phenolics and flavonoids. It is known that vitamin C (ascorbic acid) and carotenoids are chief source of discrepancy of antioxidant/ antiradical activities in plant materials. It was obvious that ethyl acetate extract of *L. siceraria* had the greatest antioxidant activity against ABTS and showed protective capacity from OH radical. These results indicated that flavonoid and phenolic compounds in the fruit of *L. siceraria* would play the protective role from free radical-induced damage.

Table-3. Free radical-scavenging ability using ABTS assay of fruit of *L. siceraria*

Different concentration of extract	**ABTS radical activity Ethyl acetate extract*L. siceraria***	**ABTS radical activity Standard Vitamin-C**
25 µl/ml	15.3±1.11	14.04±1.47
50 µl/ml	30.6±1.20	29.52±1.58
75 µl/ml	47.1±2.36	44.23±1.69
100 µl/ml	72.6±1.44	65.23±2.1
EC_{50} Value	61.23	72.32

[a]Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

INHIBITION OF LIPID PEROXIDATION ACTIVITY

Inhibition of lipid peroxidation activity of ethyl acetate extracts from the fruit of *L. siceraria* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in ethyl acetate extract of *L. siceraria* 67.32 and lowest inhibition percentage ascorbic acid 55.63 (Table-4). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Normally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free

radicals and preventing decomposition of hydroperoxides into free radicals. Ethyl acetate extracts of fruit of *L. siceraria* fractions significantly inhibited the degree of lipid peroxidation.

Table-4.Inhibition of lipid peroxidation activity fruit of *L. siceraria*

Different concentration of extract	Inhibition % of Lipid peroxidation activity by Ethyl acetate extract	Standard Vitamin-C
25 µl/ml	25.23±1.69	17.60±2.36
50 µl/ml	42.14±1.63	21.90±1.57
75 µl/ml	58.16±2.30	33.30±2.43
100 µl/ml	67.32±2.56	55.63±0.87
EC_{50} Value	71.32	82.31

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

SUPEROXIDE SCAVENGING ASSAY ACTIVITY OF FRUIT OF *L. SICERARIA*

Ethyl acetate extracts of fruit of *L. siceraria*e exhibited powerful scavenging activity for superoxide radicals in a concentration dependent process than positive control. Ethyl acetate extract of *L. siceraria* showed highest radical activity in the percentage of 75.12 when compared to positive control 71.34 (Table-5). One of the standard method to produce Superoxide radicals is through photochemical reduction of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. These superoxide radicals are extremely toxic and may be produced either through xanthine activity or through mitochondrial reaction. Superoxide radicals are reasonably a weak oxidant may decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals. Removal of superoxide in a concentration dependent manner by ethyl acetate extracts of fruit of *L. siceraria*may be attributed to the direct reaction of its phytomolecules with these radicals or inhibition of the enzymes (Table-5). Since here, SO radicals are being generated through the non-enzymatic method, the action of ethyl acetate fraction of *L. siceraria* was a clear indication of its direct reaction with this radical species. In the past also Munasinghe et al., (2001) have shown the antioxidant potential of the total alcoholic extract of root of *Vitexnegundo* on different systems, which supports our findings. However no such finding on superoxide is reported with leaf extract.

Table-5. Superoxide scavenging assay activity of fruit of *Lagenaria siceraria*

Different concentration of extract	Superoxide scavenging activity Ethyl acetate extract fruit of *L. siceraria*	Standard Vitamin-C
25 µl/ml	22.23±1.46	16.37±0.82
50 µl/ml	31.25±2.36	27.35±1.89

75 µl/ml	52.14±2.60	46.32±2.37
100 µl/ml	75.12±2.54	71.34±0.25
EC_{50} Value	64.32	69.32

[a] Results are expressed as percentage of Superoxide scavenging activity with respect to control. Each value represents the mean+SD of three experiments.

NITRIC OXIDE RADICAL SCAVENGING ASSAY

Nitric oxide radical quenching activity of the aqueous and ethyl acetate extracts of fruit of *L. siceraria* were identified and compared with the standard ascorbic acid. The ethyl aceate fraction of *L. siceraria* displayed the maximum inhibition of 76.32% at a concentration of 100 µg/ml, in a concentration-dependent process when compared to ascorbic acid with inhibition percentage (Table-6). In the current study, nitrite was produced by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by aqueous and ethyl acetate extracts. Significant scavenging activity may be due to the antioxidant property of flavonoid, which compete with oxygen to react with nitric oxide, leading to less production of nitric oxide. Nitric oxide is a potent pleiotropic mediator of physiological processes, such as smooth muscle relaxation, neuronal signalling and inhibition of platelet aggregation and regulation of cell-mediated toxicity. The scavenging activity of the by Aqueous and ethyl acetate extracts of fruit of *L. siceraria* against nitric oxide was detected by its ability to inhibit the formation of nitrite through direct competition with oxygen and oxides of nitro-gen in the reaction mixture (Alasalvar et al., 2006).

Table-6. Nitric oxide radical scavenging assay of the aqueous and ethyl acetate extracts of fruit of *L. siceraria*

Different concentration of extract	**Nitric oxide radical activity**	
	Ethyl acetate extract fruit of *L. siceraria*	**Metal chelating activity Standard Vitamin-C**
25 µl/ml	22.34±2.87	19.32±0.89
50 µl/ml	36.32±1.83	33.34±1.78
75 µl/ml	58.34±2.37	53.67±2.34
100 µl/ml	76.32±2.48	72.37±2.47
EC_{50} Value	61.32	66.38

[a] Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of two experiments.

DIETARY CONSTITUENTS OF FRUIT OF *L. SICERARIA*

The results of the macro nutrients composition and energetic value obtained for the studied vegetable varieties are shown in Table-. Moisture ranges between 92.52 g/100 g fw in the fruit of *L. siceraria*. The highest levels of protein and ash were found in the L. siceraria 0.78. Otherwise, this sample gave the lowest fat levels (0.03 g/100 g fw). Carbohydrates were the most abundant macronutrients and the highest levels were also found in the *L. siceraria* (6.89 g/100 g fw).

Table-7. Dietary constituents of fruit of *L. siceraria*

S.No.	Constituents	Fruit of *L. siceraria* (g/100g of dry)
1	Total sugar	6.870
2	Reducing sugar	6.420
3	Non-reducing sugar	0.750
4	Starch	1.450
5	Crude fiber	4.450
6	Neutral detergent fiber	20.610
7	Acid detergent fiber	17.350
8	Hemi cellulose	6.240
9	Cellulose	16.070

CONCLUSION

Polyphenols are valuable green vegetables constituents for the scavenging of free radicals because of their phenolic hydroxyl groups. This, together with the obtained results, suggests that as the amount of polyphenolic compounds increases, the antioxidant activity also increases. In conclusion, the present study demonstrates that the ethyl acetate extract of fruit of *L. siceraria* can protect the body from oxidative stress from ROS, which may be due to the phyto-chemicals in the form of polyphenols that occur in the plant. These may be used in nutraceuticals and the food industry. However, additional studies are necessary to develop a method for the fractionation and identification of polyphenols and to determine the most active antioxidant compounds in the polyphenol aqueous and ethyl acetate extracts.

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