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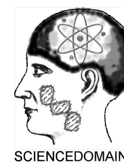

Antioxidant Capacity of Phenolic from Seed Extracts of *Lagenaria siceraria* (Short-Hybrid Bottle Gourd)

Bassey S. Antia[1], Emmanuel E. Essien[1*] and Blessing I. Udoh[1]

[1]Department of Chemistry, University of Uyo, Akwa Ibom State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors BSA and EEE designed the study, wrote the protocol, and the first draft of the manuscript. Author BIU conducted experimental work, managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the antioxidant components and antioxidant activities of seed extracts of *Lagenaria siceraria* (Short-Hybrid Bottle Gourd).
Study Design: *In vitro* assessment of antioxidant assays; quantitative determination of phenolic phytochemicals in seed extracts of short-hybrid bottle gourd.
Place and Duration of Study: Department of Chemistry, Faculty of Science and Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria (September 2013 –July 2014).
Methodology: Standard methods were used to evaluate the concentration of total phenols, total flavonoids, β-carotene, tannins, vitamin C and vitamin E in the seed extracted with different polar solvents (diethyl ether, chloroform, ethyl acetate, *n*- butanol, methanol and water). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging activities, iron chelating activity and ferric reducing antioxidant power (FRAP) were also determined using standard methods.
Results: The total phenols content ranged from 30.1-63.1 µgGAE/g, total flavonoids (30.1-49.2 µgQE/g), carotenoids (0.071-2.599 mg/100 g), tannins (7.0-25 mg/100 g), vitamin C (2.5-12.0

**Corresponding author: E-mail: emmaflowus1@yahoo.co.uk;*

mg/100 g) and vitamin E (5.5-25 mg/100 g). *In vitro* antioxidant activity of the extracts (100 μg/ml) for DPPH scavenging assay was between 57.2-63.4% and IC_{50} (50.0- 85.0 μg/ml); metal chelating (49.0–59.0%) and IC_{50} (32.0-105.0 μg/ml); and ferric reducing capacity (0.466-0.724) and IC_{50} (24.0-115 μg/ml).
Conclusion: The seed extracts of *L. siceraria* contains substantial amount of polyphenolic compounds and exhibits significant antioxidant activity. The results of this study substantiates the role of these seeds as natural sources of antioxidants which could be further exploited for their potential biological activity in related cultivars which hitherto were scarcely used as soup thickener and for dough, cakes and edible oils.

Keywords: Cucurbitaceae; Lagenaria siceraria; phenolic compounds; antioxidant activity.

1. INTRODUCTION

In biological systems, free radicals are produced during oxidation and through exposure to toxins and pollutants. Free radicals are groups of atoms having unpaired electrons which include hydroxyl, peroxyl and superoxides. Interaction of free radicals with surrounding molecules lead to the production of reactive oxygen species (ROS) which have been linked to severe diseases like cancer, cardiovascular diseases (astereosclerosis and stroke), neurological disorder, Alzheimer's disease, aging, diabetes mellitus, renal disorder, gastric ulcers, among others [1]. In recent years, antioxidants have received a great deal of attention mainly on their role in preventing diseases caused as a result of oxidative stress. Antioxidants provide protection to living organisms from damage caused by excessive production of ROS and concomitant lipid per-oxidation, protein damage and DNA stand breaking [2]. The biological effects of plants derived products in the treatment of diseases have gained a lot of interest due to their potent antioxidant activities, economic viability and absence of side effects [3].

Phenolics are the most widespread secondary metabolite in plant kingdom. Phenolic compounds such as flavonoids, phenolic acid and tannins possess diverse biological activities which might be related to their antioxidant activity [4]. They are known as powerful chain breaking antioxidant. These groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as efficient radical scavengers and metal chelators. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers [5].

Lagenaria siceraria (Molina) Standley, commonly known as bottle gourd is a large pubescent climbing herb which belongs to Cucurbitaceae family. The family Cucurbitaceae consists of 118 genera and 825 species. These species are widely distributed in the world. Many of them are economically important domesticated species and some have nutritional and therapeutic potentials [6]. In some countries like Niger, some Cucurbitaceae seeds are used for dough, cakes and as edible oils [7]. The young fruit of *Lagenaria siceraria* is also reported to be a good source of vitamin B complex, vitamin C, β-carotene and choline [8,9]. Ethnomedicinal usage includes, analgesic, antihyperglycemic, antihyperlipidemic, anti-inflammatory, antibacterial and diuretic. Lagenin- a ribosome inactivating protein isolated from the seeds of *L. siceraria* possessed antiproliferative, immuno-protective, anti HIV and antitumor properties [10]. Essien et al. [11,12] reported the oils quality and fatty acids profile of several cultivars of *L. siceraria* endemic to Nigeria. Ajani et al. [13], also evaluated the acute and sub-acute toxicity effects of ethanolic leaves extract of *L. brevifolia* (bitter gourd) on hepatic and renal function of rats. However, there has been a paucity of scientific information on their chemical constituents and biological activities. The present study was undertaken to evaluate the phenolic phytochemicals and *in vitro* antioxidant activity of seeds obtained from mature fruits of *L. siceraria* (short-hybrid bottle gourd).

2. MATERIALS AND METHODS

2.1 Sample Collection, Preparation and Extraction

The mature fruits of cultivated *L. siceraria* were collected from a farmland in September, 2013 at Ikono Local Government Area of Akwa Ibom State, Nigeria. The plant was identified and authenticated by Dr. M. E. Bassey, a taxonomist of the Botany and Ecological Studies Department, University of Uyo, where voucher specimens were deposited.

The mature fruits were beaten and allowed to soften after which the seeds were removed. The seeds were washed, shade dried for three days and pulverized with the husk. The sample (804.4 g) was extracted using cold maceration method in a jar with dichloromethane for 72 hours to remove the seed oil. The dry residue (630.8 g) was further macerated in methanol for 72 hours. The total methanol extract (41.90 g) was further partitioned with solvents of increasing polarity: diethyl ether, chloroform, ethyl acetate, and *n*-butanol. The mixture was filtered and the crude extracts/ fractions were concentrated in vacuo and dried with silica gel in a dessicator. The dried crude extracts were then weighed and the percentage yield determined. The water extract was prepared by macerating the dry residue (403 g) obtained after treatment with methanol in distilled water. All chemicals and solvents used were of analytical grade from Sigma-Aldrich GmbH, Sternheim, Germany.

2.2 Determination of Vitamin E

Vitamin E content of each of the extract was determined spectrophotometrically according to the method given by Rutkwowski and Grzegorzyk [14].

2.3 Determination of Tannin

The Tannin content in each of the extract was analysed using the method described by Bohm and Kocipai-Abyazan [15].

2.4 Determination of Carotenoids

The quantitative contents of carotenoids in the seed extracts were determined by the colorimetric method described by Nagata and Yamashita [16].

2.5 Determination of Vitamin C

Vitamin C content in the extract was determined using colorimetric method developed by Klein and Perry [17].

2.6 Determination of Phenolics

The amount of phenolics in each of the extract was determined with Folin-Ciocalteu reagent using the method of Singleton and Rossi [18]; Spanos and Wrolstad [19].

2.7 Determination of Flavonoids

Aluminium chloride colorimetric method described by Park et al. [20].

2.8 DPPH Radical Assay

DPPH (0.1 mM) in methanol was prepared and 1.0 ml of this solution was mixed with crude extract (1.0 ml) prepared in methanol at different concentrations (20, 40, 60, 80 and 100 µg/ml). (Lower absorbance of the reaction mixture indicated higher free- radical scavenging activity). The ability to scavenge DPPH radical was calculated using the equation:

$$\text{DPPH- Scavenging effect (\%)} = [(A_{control} - A_{sample}/A_{control}) \times 100]$$

IC_{50} values were calculated from the graph. Lower IC_{50} value indicated strong free radical scavenging activity. Ascorbic acid was used as positive control [21].

2.9 Metal Chelating Activity

Each crude extract (0.5 g) was mixed with $FeCl_3$ (2 mM) and ferrozine (0.2 ml) in a test tube. The total volume was diluted with methanol (2 ml). The mixture was shaken vigourously and left standing for 10 mins at room temperature. The absorbance of the solution was measured spectrophotometrically at 562 nm after the mixture had reached equilibrium using EDTA as a positive control. The percent inhibition of ferrozine-Fe^{2+} complex was calculated using the formula [22].

$$\text{Percent Scavenging} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Where:

$A_{control}$ = absorbance of ferrozine- Fe^{2+} complex
A_{sample} = absorbance of test compound

2.10 Ferric Reducing Assay

Sample solutions at different concentrations (20, 40, 60, 80 and 100 µg/ml) were mixed with phosphate buffer (0.2 M, 2.5 ml, pH 6) and potassium ferric cyanide (2.5ml, 1%). The mixture was incubated at 50ºC for 20 min, TCA (2.5 ml, 10%) was added and the mixture was centrifuged at 100ºC for 10 min. Supernatant (upper layer) (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%)

and the absorbance was measured at 700 nm [23].

3. RESULTS AND DISCUSSION

The concentration of antioxidant components are shown in Table 1. The total phenolic content ranged from 30.1-63.1 µg GAE/g. Variation in the phenolic contents of extracts may be attributed to the polarities of different solvents as well as the chemical nature of the endogenous extractable compounds. High total phenolic content for ethanol extract (121 µg/g) and methanol leaves extract (99.09 µg/mg) of *L. siceraria* was obtained by Tapkir et al. [24] and Sharma et al. [3] respectively. Phenolics exert their antioxidant activity mainly by free radical scavenging. Flavonoids recorded its highest content in the methanol extract (49.2 µgQE/g) and the lowest in diethyl ether extract (21.7 µgQE/g). This result is in congruence with our earlier preliminary phytochemical screening which revealed that *L. siceraria* (short-hybrid bottle gourd) seed extracts contain high amount of flavonoids and other phenolics in the methanol extract relative to diethyl ether, chloroform, ethyl acetate, butanol and aqueous extracts. Sharma et al. [25] and Tapkir et al. [24] reported 10 µgQE/g and 17.9 mg/g of flavonoids respectively. Flavonoids exert their antioxidant activity by scavenging free radicals, chelating metals and inhibiting lipid peroxidation. The –OH at C3 of the flavonoid structure plays a role in chelating and scavenging activity [26].

Vitamin E as an antioxidant protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction [27,28]. Vitamin E exerts it antioxidant activity by donating the hydrogen or the hydroxyl group of its chroman ring to neutralize the free radical [29]. The values for vitamin E in this work varied between 5.5-25.0 mg/100g. However, lower values (1.0 mg/100 g) and (3.39 mg/100 g) were reported by Milind and Satbir [30] and Peter et al. [31] for Indian *L. siceraria* seeds. The daily allowance of 15 mg of Vitamin E is recommended for adult. Vitamin C content in ethyl acetate extract (12 mg/100 g) was the highest and that of diethyl ether extract (2.5 mg/100 g) was the least. This result is close to the values 1.90 mg/100g and 2.94 mg/100g obtained by Miland and Satbir [30] and Peter et al. [31] respectively for seeds of *L. siceraria.* The recommended daily allowance of ascorbic acid is 60 mg (for adults) and 20 mg (for children) [32]. The body requires Vitamin C for formation of collagen, blood and hormones. It also helps in the development of bones, teeth, prevention of scurvy and as antioxidants against free radicals [33]. Vitamin C inhibits, minimizes and terminates the propagation of the free radicals by donating hydrogen and electron thus changing its structure from ascorbic acid to dehydroascorbic acid [34].

The highest value of β- carotene was seen in ethyl acetate extract (2.599 mg/100 g) and the lowest in methanol/aqueous extracts (0.071 mg/100 ml). The β-carotene in the fruit and seeds of *L. siceraria* was found to be 20 µg/100g by Morimoto and Mvere [35] and Peter et al. [31] respectively. β- Carotene is the precursor of Vitamin A. It shows antioxidant activity in humans and also plays a role in vision [36]. The mechanism by which carotenoids exert their antioxidant activity is by quenching the singlet oxygen and by trapping peroxyl radical [37,38]. Tannin exerts its antioxidant activity by scavenging free radicals, inhibiting lipid peroxidation and chelating of metal [39]. Tannins recorded its highest content in the aqueous extract (12 mg/100 g) and the lowest in ethyl acetate extract (2.5 mg/100 g). The values obtained were close to the value reported by Olaofe and Adeyeye [40] for seed flour of *L. siceraria* (9.20 mg/100 g). Tannins are known to be useful for the prevention of cancer as well as treatment of inflamed or ulcerated tissues. Tannins have astringent properties, hasten the healing of wounds and inflamed mucous membrane [41].

The results of the antioxidant activities are displayed in Figs. 1, 2 and 3. It was observed that *L. siceraria* seed extracts (20-100 µg/ml) significantly scavenged the DPPH radical in a concentration dependent manner (Fig. 1). The result in Fig. 1 also showed that at 100 µg/ml dose, the methanol extract inhibited DPPH radical by 63.4% compared to the standard drugs-BHA (75.4%) and ascorbic acid (74.2%). DPPH radical is commonly used as substrate to evaluate antioxidant activity; it is a useful and stable free radical that can accept an electron or hydrogen radical to become a stable molecule. In DPPH radical scavenging assay, the antioxidants react with the stable free radical DPPH and convert it to 1,1-diphenyl-2-picryl hydrazine with decoloration. All of the assessed extracts of *L. siceraria* seeds were able to reduce the stable, purple-coloured radical DPPH to the yellow-coloured DPPH-H form. The result in this study is similar to the data (65.93%, 100 µg/ml) and

(62.78%, 100 µg/ml) reported for methanol extract [3] and ethanol extract [42] of seeds of Indian grown *L. siceraria* respectively. However, relatively lower DPPH scavenging activity (91.25%, 60 mg/ml) was observed by Deore et al. [43] for fruit extract of *L. siceraria*. The result of DPPH scavenging activity assay in this study indicates that the seeds contain high amount of antioxidant as revealed in the pronounced free radical scavenging activity. This was more prominent in the methanol extract (63.4%, 100 µg/ml) with corresponding highest phenolic and flavonoid contents (63.1 µgGAE/g and 49.2 µgQE/g respectively). This suggests that the plant extracts contain compounds capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. Sharma et al. [3] reported IC_{50} value of 73.98 µg/ml which is in consonance with the values from this work (50.5-85.0 µg/ml) (Table 2). The IC_{50} of the extracts were compared with those expressed by ascorbic acid (23.0 µg/ml) and BHA (19.0 µg/ml) and it revealed that none of the extracts showed better antioxidant properties than the synthetic antioxidants.

The result of metal chelating activity of *L. siceraria* seeds extracts is presented in Fig. 2. The percentages of metal chelating capacity (100 µg/ml) of seed extracts of *L. siceraria* range from 49- 59%. As shown in Fig. 2, the formation of Fe^{2+} Ferrozine complex is not complete in the presence of the *L. siceraria* seeds extracts indicating that these extracts chelate the iron.

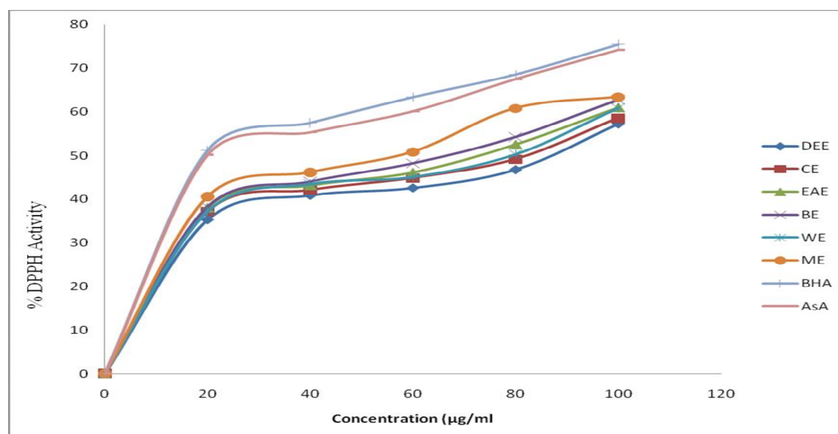

Fig. 1. DPPH scavenging activity of *L. siceraria* seeds extracts
DEE: Diethyl ether, CE: Chloroform, EAE: Ethyl acetate, BE: n-Butanol, WE: Water, ME: Methanol, AsA: Ascorbic acid

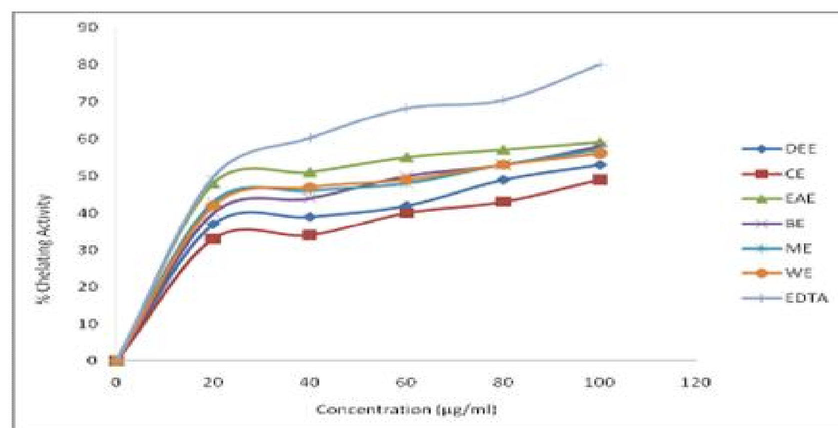

Fig. 2. Metal chelating activity of *L. siceraria* seeds extracts
DEE: Diethyl ether, CE: Chloroform, EAE: Ethyl acetate, BE: n-Butanol, WE: Water, ME: Methanol, AsA: Ascorbic acid

The result of the metal chelating activity reveals that the seeds contain antioxidants which act as chelating agent that disrupt the formation of ferrocene- Fe^{2+} complex in the solution. This is more pronounced in the methanol extract (59%, 100 µg/ml) with corresponding highest phenolic content (63.1 µgGAE/g). Transition metal ions, especially iron can stimulate lipid peroxidation by Fenton reaction and can also accelerate lipid per oxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction. Metal ion chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation. Ferrozine can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals [44].

The result obtained in this work is lower compared to data reported by Mayakrishnan et al. [45] for ethanol extract of young *L. siceraria* fruits (89.21%). IC_{50} value (Table 2) of the extracts for chelating activity was between 32.0 µg/ml and 105.0 µg/ml which are higher than the positive standard EDTA with IC_{50} of 17.5 µg/ml. The effectiveness of antioxidant properties is inversely related with IC_{50} values.

Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant. In the reducing power assay, the presence of antioxidants in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Dose response curves for the reducing powers of the seeds extracts of *L. siceraria* are shown in Fig. 3. It was found that the reducing powers of all the extracts also increased with the increase in their concentrations (20–100 µg/ml). All extracts showed good reducing power that was comparable with ascorbic acid. Methanol extract gave the highest reducing activity (0.724 at 100 µg/ml). The least activity was exhibited by chloroform extract (0.466 at 100 µg/ml) (Fig. 3). The IC_{50} (Table 2) for ferric reducing activity ranged from 24.0 to 115 µg/ml. Lower absorbances (0.305 at 1 mg/ml, 0.969 at 5 mg/ml) were reported by Mayakrishnan et al. [45]; 0.12 and 0.22 at 20 and 60 mg/ml by Deore et al. [43] for ethanolic fruit extracts of *L. siceraria*. This study suggests that the antioxidant activity of *L. siceraria* seeds extracts may be attributed to the reduction of free radicals, chelation of metal ions, or a combination thereof due to the presence of phenolic phytoconstituents.

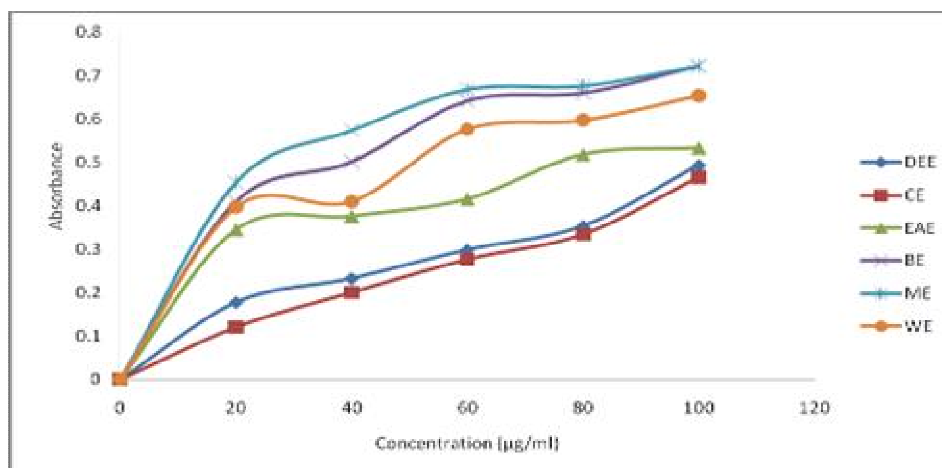

Fig. 3. Ferric reducing power activity of *L. siceraria* extracts

DEE: Diethyl ether, CE: Chloroform, EAE: Ethyl acetate, BE: n-Butanol, WE: Water, ME: Methanol, AsA: Ascorbic acid

Table 1. Antioxidant compounds in *L. siceraria* seeds extracts

Phenolic phytochemical	Diethyl ether	Chloroform	Ethyl acetate	n-Butanol	Methanol	Water
Total phenol (µgGAE/g)	30.1±0.74	33.2±0.74	60.0±0.20	42.0±0.20	63.1±1.32	60.4±0.18
Total flavonoids (µgQE/g)	21.7±0.41	31.2±0.46	41.4±0.42	32.0±0.22	49.2±0.39	44.0±0.10
Vitamin E (mg/100 g)	22.5±0.10	16.5±0.65	6.0±0.22	25.0±0.40	5.5±0.05	20.5±0.18
Carotenoids (mg/100g)	0.836±0.18	0.377±0.09	2.599±0.19	0.535±0.05	0.071±0.01	0.071±0.01
Tannins (mg/100g)	15.0±0.25	10.0±0.20	7.0±0.07	22.0±0.20	13.0±0.20	25.0±0.21
Vitamin C (mg/100g)	2.5±0.23	8.0±0.20	12.0±0.40	8.5±0.18	6.8±0.42	2.7±1.20

Data were expressed as means ± standard deviation of triplicate experiments

Table 2. IC_{50} in µg/ml for antioxidant activity of *L. siceraria* seeds extracts

Activity	Diethyl ether	Ethyl acetate	n-butanol	Methanol (µg/ml)	Water	Chloroform	EDTA	BHA	Asco-rbic acid
DPPH	79.2	68.0	70.0	50.5	67.0	85.0		19.0	23.0
Metal chelating	87.0	60.5	65.0	32.0	80.0	105.0	17.00		
Ferric reducing	110	83.0	39.0	24.0	33.0	115			

4. CONCLUSION

The seed extracts of *L. siceraria* (short-hybrid bottle cultivar) contain substantial amount of phenolic compounds especially flavonoids. Carotenoids and vitamin C were predominant in ethyl acetate extract; aqueous extract contained the highest concentration of tannins and vitamin E was most abundant in n-butanol extract. The seed extracts exhibits significant antioxidant activity – DPPH radical scavenging, metal chelating and ferric reducing when compared with standard compounds. Methanol extract demonstrated the most significant antioxidant activity and also highest total flavonoids and phenolics which could be responsible for the activity.

The investigation has revealed that *L. siceraria* seeds are potential sources of antioxidant agents. Isolation, purification, identification and structure elucidation of the phenolic phytochemical constituents could lead to the discovery of new drugs that may cure many degenerative diseases.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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