In vitro analysis of anti-diabetic and anti-oxidative potential of pedicles of fruit-vegetable bottle gourd

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Abstract: The fruit-vegetable *Lagenaria siceraria* is well known for its ethnomedicinal applications. While other parts of the plant have been studied for their medicinal properties, its fruit pedicles have not been yet explored. The present study therefore aimed to investigate their phenolics, flavonoids, antioxidant potential and alpha-amylase inhibitory properties. The bioactivities of this neglected part of the fruit were promising. Ethyl acetate fraction had the highest total phenolic content (TPC), 4.4µg/mL Gallic acid equivalent (GAE). The TPC of chloroform and n-butanolic fractions were 3.6 and 2.5 GAE, respectively. Chloroform fraction displayed the highest total flavonoid content (TFC, 295µg/mL Rutin equivalent). The trend of TFC among the fractions was chloroform > hexane > ethyl acetate > *n*-butanolic > aqueous. Ethyl acetate fraction was most potent as a DPPH radical scavenger, and showed notable activity even at very low concentration (IC_{50} 2.65mg/mL). It was more potent than ascorbic acid (IC_{50} 4.9mg/mL), the standard used in the study. The methanolic extract itself was more powerful than ascorbic acid. The residual aqueous fraction was the strongest inhibitor of alpha-amylase with IC_{50} 1.35mg/mL, which was comparable to the antidiabetic drug Acarbose (IC_{50} 1.26 mg/mL). The IC_{50} (mg/mL) of ethyl acetate, hexane and *n*-butanolic fractions were 2.16,2.05 and 2.44, respectively. The findings indicated that the pedicles of *L. siceraria* fruits have remarkable antioxidant and alpha-amylase inhibitory potential. Subject to verification by *in vivo* analysis and clinical trial, consumption of the pedicles of this fruit may be advised to diabetic people. As the aqueous fraction was the most potent inhibitor, a water decoction of the fruit part may safely be recommended for the purpose.

Keywords: *Lagenaria siceraria*, bottle gourd, anti-diabetic, antioxidant.

INTRODUCTION

The plant family Cucurbitaceae (also called gourd family) is a large family. Bottle gourd, pumpkin, cucumber, melon, and watermelon are some of its common plants, which are known for their nutritional and medicinal values, and are cultivated almost throughout the world (Rai *et al*., 2008). The plant *Lagenaria siceraria* (Mol.) Standley has highly diversified ethnomedicinal applications, which in recent years has prompted a number reviews (Alamgir *et al*., 2016; Aslam & Najam, 2013; Kumar *et al*., 2012). It considered to be cardio-tonic, liver tonic, general tonic, diuretic, antibilious, and is used for treatment of pain, ulcer, fever, cough, asthma, bronchitis, leucorrhoea, and vaginal and uterine complaints. Chemical studies found *L. siceraria* to contain a variety of secondary metabolites, which include β-carotene, vitamin C, saponins, cucurbitacins, flavone-C-glycoside, dietary fibers, proteins, palmitic acid, steric acid, oleic acid, polyphenols, terpenoids and steroids (Chaudhery *et al*., 2014; Kubde *et al*., 2012; Shah *et al*, 2010).

Recently, *L. siceraria* has been investigated comprehensively for its biological activities including antioxidant potential (Ahmed *et al*., 2014a; Dar *et al*., 2014; Erasto & Mbwambo, 2009; Deshpande *et al*., 2007). The antioxidants are the substances that inhibit or scavenge toxic free radicals. The free radicals, which are formed in in our body under metabolic and environmental factors, can cause various degenerative and pathological disorders, such as cancers, coronary heart diseases and neurodegenerative disorders (Strand, 2016). As the plant-based antioxidants are considered to possess no or very little side effects, the fruit-vegetable *L. siceraria* has been studied with a view to find natural remedies for degenerative diseases. An important medicinal aspect of *L. siceraria* is its anti-hyperglycemic properties (Kumar *et al.*, 2012; Saha *et al*., 2011). Diabetes is a chronic metabolic disorder, and is classified as type-1 and type 2. The diabetes type 1 is characterized by the inability of the body to produce insulin. On the other hand, in diabetes type-2, insulin is not properly used by the body. According to WHO (World Health Organization), the global prevalence of diabetes in 2014 was 9% among people with 18+ years age. By the year 2030, the disease has been projected to be the 7th principal cause of death (WHO, 2014; Mathers *et al*., 2006).

One of the strategies that are used to control glucose level in the blood in diabetic patients employs inhibition of alpha-amylase from starch hydrolysis. Alpha-amylase in our digestive system converts starch into monosaccharides and oligosaccharides, which upon further reaction produce glucose. Glucose is then absorbed into the blood stream. Inhibitors such as

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Acarbose are available but they have side effects. This necessitates efforts to search for new drugs. A lot of hope rests with herbal products and numerous plants have been reported to have anti-diabetic effect (Ahmed *et al*., 2014b; Ali *et al*., 2006; Funke and Melzig, 2006; Kim *et al*., 2005).

Although the fruit of *L. siceraria* has been extensively studied, the literature shows no work on its pedicles. While the fruit is used as vegetable, its pedicles are just discarded. The present work, therefore, aimed to evaluated pedicles of *L. siceraria* for their total phenolic and flavonoid contents, antioxidant potential and alpha-amylase inhibitory activity.

MATERIALS AND METHODS

Chemicals and equipment

Gallic acid was purchased from RDH (Germany). Folin-Ciocalteu reagent was obtained from Merck (Germany). Ascorbic acid, Rutin, and DPPH radical were obtained from MP Biomedicals (France). Alpha-amylase and DNS (3,5-Dinitrosalicylic acid) were obtained from Unichem (Turkey). UV-Visible Spectrophotometer-UVD-3200, Labomed, Inc. was used to record absorbance.

Collection of the plant

Fresh *L. siceraria* fruits were collected from an agricultural farm of Pattoki, Pakistan. The taxonomy of the plant was verified by Dr. Khalid Zamir Rasib, Associate Professor of Biological Sciences, FC College University, Lahore, Pakistan. The plant material was washed with distilled water. The pedicles were separated, crushed, dried and ground to obtain a powder.

Preparation of methanolic extract and its fractions

Cold maceration method was used to extract phytochemicals. The pedicles powder (120g) was soaked in methanol (1L) for 15 days, with shaking from time to time. It was filtered and the filtrate was concentrated on rotary evaporator under reduced pressure to obtain methanolic extract as a gummy material. A weighed amount of the methanolic extract was mixed in distilled water and sequentially fractionated into hexane, chloroform, ethyl acetate and *n*-butanol, leaving behind residual water fraction. These fractions were weighed and stored in a refrigerator.

Total phenolic content assay

The methanolic extract of the pedicles of *L. siceraria* fruits and its fractions were evaluated to determine the total phenolic content (TPC) by following a reported method (Slinkard & Singleton, 1977) with slight modification. Gallic acid, a polyphenol, was used as a positive control. The plant samples were prepared by dissolving each extract/ fraction in 10mL methanol. In a clean test tube, 40µL plant sample (or Gallic acid solution) was diluted with 3.16mL distilled water. Folin-Ciocalteu reagent (200µL) was added. The mixture was shaken to mix thoroughly. After 8 min interval, 600µL Na_2CO_3 (0.2g/mL in water) was mixed, and the solution was incubated for 30min at 40°C. The blank contained 40µL methanol instead of a plant sample. The absorbance was recorded at 765nm. The TPC of a sample was expressed as µg/mL GAE (Gallic acid equivalent).

Total flavonoid content assay

A reported method (Ahmed *et al*., 2014c) was followed to evaluate total flavonoid content (TFC) of the methanolic extract of *L. siceraria* fruit pedicles and its fractions. Rutin was used as standard. Each plant sample was prepared in methanol (1mg/mL). To 300µL plant sample (or Rutin solution), 3.4mL aqueous methanol (30%) and 150µL $NaNO_2$ solution (0.5M) was mixed. After 5 min, 150µL $AlCl_3$ solution (0.3M) was added. It was further incubated for 5 min before adding 1mL NaOH solution (1 M). The mixture was mixed thoroughly to obtain clear solution. Absorbance was noted at 506nm. The blank contained 300µL aqueous methanol (30%) instead of a plant sample. TFC of each sample was expressed as µg/mL RE (Rutin equivalents).

DPPH radical scavenging assay

To determine antioxidant activity of the methanolic extract of *L. siceraria* fruit pedicles and its fractions, the DPPH (diphenylpicrylhydrazyl) radical scavenging assay was conducted according to a known protocol (Brand-Williams *et al.*, 1995). A stock solution of DPPH in methanol (24mg/100mL) was used to prepare its working solution. Thus, 3.5mL DPPH stock solution was diluted with methanol to attain absorbance of 0.97 (± 0.03) at 517nm. The stock solution of each plant sample was prepared in methanol (20mg/mL), from which various dilutions were made for study. Ascorbic acid was used as a standard. To the DPPH working solution (3mL), 100µL plant sample was mixed. Absorbance was recorded at 517 nm after an incubation of 30min at 37°C. The blank consisted of methanol. The negative control contained 100µL methanol instead of a plant sample. The DPPH scavenging activity of a plant sample was estimated as per the formula given below:

$$\% \text{ Radical Scavenging Activity} = \left[\frac{(A_{Control} - A_{Sample})}{A_{Control}}\right] \times 100$$

Alpha-amylase inhibitory assay

Alpha-amylase inhibitory properties of the methanolic extract of *L. siceraria* fruit pedicles and its fractions were determined according to a reported procedure (Nickavar *et al.*, 2008), explained in table 1. To prepare its solution, alpha-amylase enzyme (1mg) was dissolved in sodium phosphate buffer (pH 6.9, 100mL). Potato starch solution (0.5% in distilled water) was used as a substrate. The

DNS coloring reagent was prepared by combining 96mM 3,5-dinitrosalicylic acid solution with Na/K tartrate solution (5.3mM). Each plant sample was prepared by dissolving its 0.04g in 20mL dimethyl sulfoxide, from which further dilutions were prepared. Three test tubes were labeled as sample, blank and control. In the test tube of sample, 1mL plant sample was mixed with 1mL enzyme solution, and the mixture was incubated at 25°C for 30min. To 1mL of this mixture, 1mL starch solution was mixed followed by an incubation of 3min at 25°C. The DNS reagent (1mL) was added and mixed. The mixture so obtained was heated in a water bath at 85°C for 15min. It was allowed to come to room temperature before addition of 9mL distilled water.

In the test tube of blank, as above 1mL enzyme solution was added to 1mL plant sample. . It was incubated at 25 °C for 30min. To 1mL of this mixture, 1mL DNS reagent was added followed by an incubation for 3 min at 25°C. Then, starch solution (1mL) was added. Rest of the procedure is same as followed for sample. After heating the mixture in a water bath 85°C for 15min, it was cooled to room temperature and diluted with 9mL distilled. The control was prepared as per the method used for sample, except an equal amount DMSO replaced the plant sample. The absorbance of the sample, blank and control was noted at 540nm. The alpha-amylase inhibitory activity of a sample in percentage was calculated as per the formula shown below:

$$\%\text{Inhibitory activity} = 100\left[\frac{\text{Ac - As)}}{\text{Ac}}\right]$$

Where As and Ac are the absorbance of the plant sample and negative control, respectively.

STATISTICAL ANALYSIS

All the activities were determined at least thrice and results were expressed as mean±SD. For statistical analysis, MS Excel 2010 was used. For free radical scavenging and enzyme inhibitory activities, IC_{50} values were calculated using Biodata fit online software**.**

RESULTS

Total phenolic content (TPC) and Total flavonoid content (TFC)

Total phenolic content (TPC) and total flavonoid content (TFC) of the methanolic extract of pedicles of *L. siceraria* fruits and its fractions were evaluated and the results are exhibited in table 2.

DPPH radical scavenging activity

The DPPH radical scavenging potential of *L. siceraria* fruit pedicles was screened over a range of dilutions. The activities were found to be dose dependent. Hence, IC_{50} values were calculated, which are displayed in table 2.

Alpha-amylase inhibitory activity

Alpha-amylase inhibitory activities of the fruit pedicles of *L. siceraria* were evaluated over a range of dilutions. IC_{50} values were calculated and the results are shown in table 3. Acarbose was used as a standard.

DISCUSSION

As highlighted in the introduction, the fruit-vegetable *L. siceraria* is well-known for its ethno-medicinal properties. Hence, a number of researchers have made it a subject of their investigation (Ahmed *et al*., 2016; Alamgir *et al*., 2016; Saha *et al*., 2011). While other parts of the fruit have been studied extensively, its pedicles have not been evaluated yet for any medicinal activity. The present work aimed to study phenolics, flavonoids, and antioxidant and alpha-amylase inhibitory potential of pedicles of the fruit. Methanol was used as a solvent to extract maximum secondary metabolites. The methanolic extract was then subjected to further fractionation into solvents of increasing polarity in order to trace the bioactive compounds.

Phenolics and flavonoids are known for their antioxidant activity as they have remarkable ability to scavenge free radicals produced in our body. Therefore, an estimate of phenolics and flavonoids in a plant sample provides an indication of its antioxidant potential. As the table 2 showed, the findings of total phenolic and flavonoid contents of the extract and its fractions were interesting. Phenolic content was highest in ethyl acetate fraction, i.e., 4.4µg/mL GAE (Gallic acid equivalent). This was as per expectation as ethyl acetate is known to be a good solvent for the extraction of phenolics (Ahmed *et al*., 2014c). Chloroform fraction also showed good phenolic content (3.6µg/mL GAE) followed by *n*-butanolic fraction (2.5 µg/mL GAE). Aqueous fraction had the lowest TPC, while chloroform fraction showed the highest TFC (295 µg/mL Rutin equivalent). Trend of flavonoid content was chloroform> hexane> ethyl acetate> n-butanolic> aqueous. There is a considerable parallelism between phenolics and flavonoids (table 2).

The DPPH is a stable free radical. The test based on this radical is commonly used to evaluate antioxidant or free radical removing ability of a substance. All the plant samples showed concentration dependent activity except hexane fraction, which displayed no notable activity at any of its concentrations. The ethyl acetate fraction that exhibited maximal TPC was most potent as a DPPH radical scavenger. It showed prominent activity even at very low concentration (IC_{50}2.65mg/mL), it was more powerful than ascorbic acid (IC_{50}4.9mg/mL), the standard used in the study. The methanolic extract itself was more potent than ascorbic acid. The high antioxidant potential of the ethyl acetate fraction might be explained based on

Table 1: Protocol used to determine alpha-amylase inhibitory activity

Reagents	Control	Sample	Blank
Plant sample	-	1 mL	1 mL
DMSO	1 mL	-	-
Enzyme solution	1 mL	1 mL	1 mL
Mix and incubated at 25 ^{o}C for 30 min			
Starch solution	1 mL	1 mL	-
DNS reagent	-	-	1 mL
Mix and incubated at 25 ^{o}C for 3 min			
DNS reagent	1 mL	1 mL	-
Starch solution	-	-	1 mL

Table 2: Total phenolic (TPC), flavonoid (TFC) contents and DPPH scavenging activity of methanolic extract of *Lagenaria siceraria* pedicles and its fractions in solvents of different polarity (n = 3) nd could not be detected because of poor activity.

Extract / Fraction	µg/mL Gallic acid equivalent	µg/mL Rutin equivalent	DPPH Scavenging activity, IC_{50} (mg/mL)
Methanolic	1.8	117	2.67
Hexane	1.6	185	nd
Chloroform	3.6	295	7.06
Ethyl acetate	4.4	165	2.65
n-Butanolic	2.5	105	8.42
Aqueous	0.7	80	6.08
Ascorbic acid			4.9

Table 3: Inhibitory activity of alpha-amylase of extract of *Lagenaria siceraria* fruit pedicles and its fractions along with the standard Acarbose in terms of IC_{50} values. (n = 3)

Concentration (mg/mL)	Methanolic	Hexane	Ethyl acetate	n-Butanolic	Aqueous	Acarbose
IC_{50}	2.44	2.05	2.16	2.41	1.35	1.26

its high TPC since the phenolics are known to possess high antioxidant activity.

This activity is used as a strategy to control blood glucose level. When pancreatic alpha-amylase is inhibited in the small intestine, conversion of starch into glucoses is retarded. Consequently, less amount of glucose is formed and enters into the blood stream. In the recent years, numerous studies have been conducted on plants for their possible alpha-amylase inhibitory activity (Buchhols & Melzi. 2016; Shori, 2015; Patel *et al*., 2012). This strategy is clinically employed to control blood glucose level in patients of diabetes. With this background in mind, the present study was conducted. The highest activity was shown by residual aqueous fraction with IC_{50} 1.35 mg/mL. It was almost equivalent to the medicine Acarbose (IC_{50} 1.26mg/mL). IC_{50} (mg/mL) of *n*-butanolic, ethyl acetate and hexane fractions were 2.44, 2.16 and 2.05, respectively. The findings indicated that fruit pedicles of *L. siceraria* have remarkable potential for inhibition of alpha-amylase enzyme. Subject to verification by in vivo analysis and clinical trial, consumption of the pedicles of this fruit may be advised to diabetic people. As the aqueous fraction was the most potent inhibitor, a water decoction of the fruit part may safely be recommended for the purpose.

CONCLUSION

Study of the antioxidant and enzyme-inhibitory activities of the pedicles of *Lagenaria siceraria* fruit provided a scientific basis for the ethnomedicinal applications of this fruit-vegetable. While the ethyl acetate fraction was more potent antioxidant than ascorbic acid, the aqueous fraction showed better alpha-amylase inhibitory activity than Acarbose, a well-known medicine used as an anti-diabetic agent. Subject to verification by *in vivo* analysis and clinical trial, consumption of the pedicles of this fruit may be advised to diabetic patients. As the aqueous fraction was the most potent inhibitor, a water decoction of the fruit part may safely be recommended for the purpose.

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