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A comparative study of the antioxidant, antimicrobial, cytotoxic and thrombolytic potential of the fruits and leaves of *Spondias dulcis*

Shawkat Md. Aminul Islam, Kh Tanvir Ahmed*, Mohammad Kawsar Manik, Md. Arif Wahid, Chowdhury Shafayat Ibne Kamal

Department of Pharmacy, East West University, Aftabnagar, Dhaka-1212, Bangladesh

PEER REVIEW

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Zobaer Al Mahmud, Department of Clinical Pharmacy and pharmacology, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh.
Tel: +8801722597925
Fax: 880-2-8615583
E-mail: zalmahmud@du.ac.bd

Comments

This is a good study in which authors investigated the antioxidant, antimicrobial, cytotoxic and thrombolytic property of the fruits and leaves of *S. dulcis* using different *in vitro* biochemical analysis, disc diffusion methods and *in vivo* bioassay *etc*. The methanolic fruit extracts of the plant exhibited most promising results and might be a potential source of lead compounds for aforementioned bioactivities.

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ABSTRACT

Objective: To investigate the antioxidant, antimicrobial, cytotoxic and thrombolytic property of the fruits and leaves of *Spondias dulcis* (*S. dulcis*).

Methods: Methanolic extracts of fruits and leaves of *S. dulcis* were partitioned with chloroform and dichloromethane. The antioxidant potential of the crude extract and partitioned fractions were evaluated in terms of total phenolic content, total flavonoid content, DPPH radical scavenging potential, reducing potential and total antioxidant capacity by specific standard procedures. The antimicrobial activity was evaluated using disc diffusion method. The cytotoxicity was evaluated by using brine shrimp lethality bioassay and compared with vincristine sulfate. The thrombolytic activity was compared with streptokinase.

Results: The methanolic fruit extract exhibited the highest phenolic content, flavonoid content and antioxidant capacity, among the other extracts, with the highest DPPH radical scavenging activity at a concentration of 10 μg/mL (IC_{50}: 1.91 μg/mL) and maximum reducing power at a concentration of 100 μg/mL (EC_{50}: 3.58 μg/mL). Though all extract showed moderate antimicrobial activity against the bacterial strains, weak or no activity against fungus. The range of LC_{50} value of all extracts was 1.335-14.057 μg/mL which was far lower than the cut off index for cytotoxicity. All extracts exhibited statistically significant ($P<0.001$) thrombolytic activity.

Conclusions: Our study suggested that *S. dulcis* exhibits antimicrobial activities against a wide variety of strains while it possesses significant antioxidant, cytotoxic and thrombolytic activity.

KEYWORDS

Spondias dulcis, Antioxidant activity, Antimicrobial activity, Cytotoxic activity, Thrombolytic activity, DPPH, IC_{50}, LC_{50}

1. Introduction

The demand in study of plants, which is one of the richest sources of promising versatile chemical compounds and medicinal values, is growing constantly throughout the world during the last few decades. Therefore plant could play a great role in exploring new arsenals against the threats of new and recent diseases. Because of their potent pharmacological activity, low toxicity and economic feasibility, investigation of the medicinal properties of plant has been performed[1]. Along with the nutritional value, plants contribute in the protection from free radical deterioration by hindrance of lipid peroxidation via numerous mechanisms including

*Corresponding author: Kh Tanvir Ahmed, Department of Pharmacy, East West University, Aftabnagar, Dhaka-1212, Bangladesh.
Tel: +8801716878316, +880-2-9858261, +880-9666775577 (Ext-119)
Fax: +880-2-8812336
E-mail: kta@ewubd.edu

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scavenging free radicals[2], inducing antioxidant enzymes[3], modulating protein kinase, and lipid kinase signaling pathway[4], inhibiting cyclooxygenase-2 (COX 2) and matrix metalloproteinases (MMP 2 and MMP 9) enzymatic activity[5,6], influencing phytoestrogenic and nuclear transcription factor NF-κB[7,8], inducing cell cycle arrest and phase 1 or phase 2 metabolizing enzymes[9,10]. Oxidative damage done by free radicals causes the pathogenesis of many deadly diseases like cancer, Alzheimer's and diabetes[11]. Due to the possible toxicity of synthetic antioxidant, scientists recently have focused on using plant materials as natural antioxidant[12]. In addition to the peril multiple drug resistance resulting from the chaotic application of commercial antimicrobial drugs, adverse effects such as hypersensivity, allergic reaction, immune suppression abdominal pain, anorexia *etc*. drive the scientists to explore new and effective antimicrobial agents that could act as an alternative of the current regimens[13-15]. Cancer, a notorious disease of present time, has become the major cause of human mortality in the world, and approximately half of incidence and mortality occurs in Asia[16]. Nevertheless, accelerating numerous side effects, chemotherapeutic drugs are still regarded as the most effective treatments for cancer. Concerning the serious crisis, scientists have currently engaged in finding natural medicine resources as an alternative to current chemotherapeutic regimens. Besides, phytochemicals containing antioxidant potentials showed capacity to inhibit carcinogenesis[17]. Several studies revealed that antioxidants are related to other bioactivities *e.g. in vitro* cytotoxicity in tumor cell and *in vivo* cytotoxicity in experimental animals and anticarcinogenesis[18-20]. Almost all currently available thrombolytic regimens have some vital shortcomings including limited fibrin specificity, noteworthy bleeding tendency and large dose requirement. To counter this dilemma, extensive researches are carried out to find more effective natural resources which can be used as an alternative regimen[21-23].

The genus *Spondias* (Anacardiaceae) consists of 17 species; seven of which are native to the neotropics and about 10 are native to tropical Asia. About 10 species of *Spondias* bear edible fruits. Though *Spondias dulcis* (*S. dulcis*) is native to Melanesia through Polynesia, it is also abundant in Bangladesh. *S. dulcis* is a fast growing equatorial tree with edible fruits which is popular in Bangladesh in the name of amra while its English name is Hog pulm or golden apple. Though it is most commonly used as food source, the astringent bark is used as a remedy for diarrhea in Cambodia[24]. *S. dulcis* is also used in eyesight enhancement and eye infections[25], and the fruit is used to cure itchiness, internal ulceration, sore throat and inflammation of skin. The fruit is also used as an antidote[26]. Moreover, the polysaccharide identified from the fruits pulp has eliciting activity on peritoneal macrophages[27]. Furthermore, a study conducted using leaves of the plant has shown antidiabetic activity[28]. To date, there is no study comparing the antioxidant, antimicrobial, cytotoxic and thrombolytic potential of various extracts of fruit and leaves of *S. dulcis*. The report of previous studies conducted using various parts of *Spondias pinnata* influenced us to explore similar type of activity from *S. dulcis*.

Therefore, the present study was conducted on different extracts (methanol, chloroform, and dichloromethane) of fruit and leaves in order to evaluate the plant as a source of natural antioxidant, antimicrobial, cytotoxic and thrombolytic.

2. Materials and methods

2.1. Plant collection

Fruits and leaves of *S. dulcis* were collected in August 2011, from Mirzapur (Tangail), Bangladesh. The fresh leaves were then thoroughly washed to remove soil and dirt while the peel and seed of the fruits were removed. Both the leaves and chopped fruits were then shade dried for 2 weeks followed by grinding into fine particles. They were then kept in two separate closed containers at room temperature until further used. The plant was identified at Bangladesh National Herbarium where a voucher specimen was deposited.

2.2. Extraction

The pulverized plant materials were soaked in 3.5 L of methanol in separate containers and kept for 1 week with occasional shaking. The whole mixtures were then filtered through sterilized cotton followed by Whatman No. 1 filter paper and concentrated using a rotary evaporator (IKA, Germany). The weight of crude extracts of fruits and leaves were 135 g and 101 g, respectively. A total of 40 g crude extract of each plant materials was then partitioned with chloroform and dichloromethane. The percentage yields of fruit and leaves were methanol (21.3%, 15.8%), chloroform (19.5%, 23.1%) and dichloromethane (20.9%, 25.4%) respectively.

2.3. Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, gallic acid, quercetin and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, USA). Analytical grade dichloromethane, methanol, ethanol and chloroform were purchased from BDH, E Merck. Vincristine Sulphate was purchased from Cipla Ltd., Goa, India. Cephradin and nystatin were purchased from Square Pharmaceuticals Ltd., Bangladesh. Streptokinase was purchased from Beacon pharmaceutical Ltd., Bangladesh. All other chemicals and reagents of analytical grade including methanol and dimethyl sulfoxide (DMSO) were from Merck (Germany).

2.4. Phytochemical screening

The freshly prepared crude extracts of the fruit and leaves of *S. dulcis* were qualitatively tested for the presence of alkaloids (Dragandroff's reagent), flavonoids (cyanidine reaction), steroids (Salkowski test), terpenoids (Modified Salkowski test), reducing sugars (Fehling reagent), saponins (Frothing test), tannins (iron chloride), cardiac glycosides (Keller-Killani test) and anthraquinones (Chloroform layer test)[29-31].

2.5. Antioxidant activity

2.5.1. Total phenolic content

The total phenols in extracts and fractions of *S. dulcis* were determined using Folin-Ciocalteu reagent[32]. About 0.5 mL of the each extract of concentration of 1 mg/mL was mixed with 5 mL Folin ciocalteu reagent (1:10 v/v distilled water) and 4 mL (75 g/L) of sodium carbonate. The mixture was allowed to stand for 30 min at room temperature in dark place after vortexing it for 15 seconds and the absorbance was measured at 760 nm against methanol as blank by using a UV-visible spectrophotometer. The total phenolic contents were expressed as gallic acid equivalent (GAE) in mg/g of dry weight. For correlation study between total phenolic content and total antioxidant activity, the whole procedure was repeated to determine the phenolic content of various sample concentration (0.1, 0.2, 0.4, and 0.8 mg/mL).

2.5.2. Total flavonoid content

The total flavonoid in each fraction of *S. dulcis* was measured by aluminum chloride ($AlCl_3$) colorimetric method[33]. About 0.5 mL methanol solution of each extract of concentration of 10 mg/mL was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 mol/L potassium acetate and 2.8 mL of distilled water. The blank was prepared in similar fashion by replacing $AlCl_3$ with distilled water. Both sample and blank solution were filtered through double rings filter paper. After an incubation period of 30 min, the absorbance was measured at 415 nm against the blank by using a UV- visible spectrophotometer. The total flavonoid contents were expressed as quercetin equivalent in mg/g of dry weight.

2.5.3. DPPH free radical scavenging activity

The radical scavenging activity of each fraction of *S. dulcis* was estimated using stable free radical of DPPH[34]. About 2.0 mL of methanol solution of each extract at different concentration (2, 4, 6, 8, 10 μg/mL) were mixed with 3.0 mL of DPPH methanol solution (20 μg/mL). After an incubation period of 30 min, the absorbance was measured at 517 nm against methanol as blank by using a UV-visible spectrophotometer. The radical scavenging activity (%) was calculated based on the following formula:

DPPH scavenging activity (%)=$[(A_B-A_T)/A_B]\times100$

Where A_B and A_T are the absorbance of blank and plant material, respectively.

The percentage scavenging activity of each extract was compared with L-ascorbic acid, the positive control. IC_{50} value of each extract was determined from the plotted graph of percentage DPPH neutralization *vs.* concentration of extract, which was defined as the amount of antioxidant required to reduce the initial DPPH free radical concentration by 50%.

2.5.4. Reducing power assay

The reducing power of all the extracts of *S. dulcis* were measured by the method previously described by Oyaizu[35]. About 1 mL of each extract of varying concentrations (1, 5, 10, 50, 100 μg/mL) in double distilled water was mixed with 2.5 mL phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min after which, 2.5 mL of 10% trichloroacetic acid (TCA), was added and centrifuged at 3 000 r/min for 10 min. From each tube, 0.5 mL of the supernatant was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride. Incubation with water in place of additives was used as blank while L-ascorbic acid was used as positive control. The absorbance was measured at 700 nm by using a UV-visible spectrophotometer. Increased absorbance of the reaction mixture suggests increasing reducing power. The reducing power (%) was calculated based on the following formula:

Reducing power (%)=$[(A_T-A_B)/A_B]\times100$

Where A_B and A_T are the absorbance of blank and plant material, respectively.

Effective concentration, EC_{50} value of each extract was estimated from the plotted graph of percentage reducing power *vs.* concentration of extract.

2.5.5. Total antioxidant capacity

The total antioxidant capacity of all the extracts of *S. dulcis* were estimated by phosphomolybdenum method[36]. About 0.2 mL of each extract of concentration of 0.5 mg/mL was mixed with 2 mL reagent solution (600 mmol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The reaction mixtures were then incubated at 95 °C for 1 h. The absorbance was measured at 695 nm against a blank containing 3 mL reagent solution by using a UV-visible spectrophotometer. The total antioxidant activity of the crude extract was expressed as gallic acid equivalents in mg/g of dry weight. For correlation study, the whole procedure was repeated to determine the total antioxidant capacity of various sample concentration (0.1, 0.2, 0.4, and 0.8 mg/mL).

2.6. Antimicrobial activity

A total of 13 reference microbial strains (four Gram-positive, seven Gram-negative and two fungi) were used as

the test organism for the antimicrobial screening of all the fruits and leaves extracts of *S. dulcis*. The antimicrobial activity of the plant extracts against the test organisms was performed by disc diffusion method using standard disc (30 μg/disc) for comparison[37]. Cephradin and nystatin were used as the standard disc for comparing antibacterial and antifungal activity, respectively. The test organisms were inoculated on 10 mL previously sterilized nutrient agar media, mixed thoroughly and transferred immediately to the sterile petri dish under an aseptic condition using a sterile loop. The paper discs containing the sample extract and standard disc were placed to the corresponding petri dish and were incubated for overnight at 37 °C. Clear zone of inhibition around the discs represented the presence of antimicrobial activity which was measured in millimeter (mm).

2.6.1. Collection of microorganisms

The microbial species used in the present study were *Bacillus cereus*, *Bacillus subtilis*, *Sarcina lutea*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysentery*, *Pseudomonas aeruginosa*, *Vibrio mimicus*, *Candida albicans*, and *Sacharomyces cerevacae*. These were collected as pure cultures from the Institute of Nutrition and Food Sciences, Dhaka University and International Center for Diarrheal Disease and Research, Dhaka, Bangladesh.

2.7. Determination of cytotoxicity

The cytotoxic potentiality of all the extracts of *S. dulcis* were performed on brine shrimp nauplii using Mayer's method[38,39]. The eggs of brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank containing 1 L of simulated seawater at a temperature around 37 °C and pH 8.4 with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Pure DMSO solutions of the extracts were applied to *Artemia salina* in a one-day *in vivo* assay. About 4 mg of each extracts was dissolved in DMSO and solutions with varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 μg/mL) obtained by serial dilution technique. The prepared test solutions were added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 mL simulated seawater and incubated for 24 h. After incubation period, the vials were examined using a magnifying glass in order to count the number of survived nauplii in each vial. From this data, the lethality percent of the brine shrimp nauplii was calculated for each concentration. The median lethal concentration, LC_{50} and LC_{90} of each tested sample was calculated from the plotted graph of percentage of the shrimp mortality *vs.* logarithm of the sample concentration, which was defined as the amount of extract required to kill 50% of brine shrimps and the amount of extract required to kill 90% of brine shrimps within 24 h of exposure respectively.

2.8. Thrombolytic activity

The *in vitro* thrombolytic potential of each extract of *S. dulcis* was evaluated with the method developed by Daginawala using streptokinase as the standard substance[40]. A total of 8 mL venous blood was drawn from six healthy volunteers (three male and three female) without a history of oral contraceptive or anticoagulant therapy. Blood from each volunteer was distributed in eight different pre-weighed sterile microcentrifuge tube and incubated at 37 °C for 45 min. After clot formation, serum was completely aspirated out without disturbing the clot formed and the weight of clot in each tube was measured. To each microcentrifuge tube containing pre-weighed clot, 100 μL aqueous solution of different extracts with the concentration of 10 mg/mL was added separately. Then, 100 μL of streptokinase (SK) and 100 μL of distilled water were separately added to the control tube as positive and negative controls, respectively. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, the released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference in weight before and after clot lysis was expressed as percentage of clot lysis as shown below:

% of clot lysis = (weight of released clot /clot weight)×100

2.9. Statistical analysis

All values were expressed as mean±SD of three parallel determinations. The median lethal concentration, LC_{50}, LC_{90} and 95% confidence interval of test sample were calculated using probit analysis method described by Finney as a measure of toxicity of the plant extract. All values of thrombolytic activity were calculated as mean±SEM of three parallel determinations and were evaluated using *t*-test. $P< 0.001$ was regarded as statistically significant.

3. Results

3.1. Phytochemical screening

Phytochemical screening of the crude extract revealed the absence of anthraquinone and reducing sugar in fruits while only anthraquinone was absent in leaves. The phytochemical screening of the crude extracts is described in Table 1.

3.2. Total phenolic and flavonoid content

Table 2 provides information about the total phenolic and falvonoid content in the six extracts of fruits and leaves considered in this study. The highest phenolic and flavonid content were found in the methanolic extract of fruit

Table 1

Phytochemical screening of the crude extract of fruit and leaves of *S. dulcis*.

Sample	Anthraquinone	Saponin	Steroid	Alkaloid	Cardiac Glycoside	Tannin	Flavonoid	Terpenoid	Reducing sugar
Fruit	−	+	+	+	+	+	+	+	−
Leaf	−	+	+	+	+	+	+	+	+

Table 2

Total phenolics, flavonoids content and antioxidant activity of the different extracts of *S. dulcis*.

Sample	Extract	Total phenolics (mg/g of dry weight)	Total flavonoids (mg/g of dry weight)	Total antioxidant activity (mg/g of dry weight)
S. dulcis fruit	Methanol	659.74±0.97	225.60±0.88	118.46±0.48
	Chloroform	214.59±0.65	51.79±0.21	19.27±0.82
S. dulcis leaf	Dichloromethane	443.97±0.77	124.21±0.27	63.45±0.69
	Methanol	16.35±0.50	33.96±0.41	2.98±0.70
	Chloroform	22.62±0.80	28.21±0.63	3.46±0.32
	Dichloromethane	35.44±0.27	47.76±0.43	5.69±0.61

which amounted to (659.74±0.97) GAE/g of dry weight and (225.60±0.88) mg quercetin equivalents/g of dried weight, respectively. Among the leaf extracts, dichloromethane fraction was found to contain the highest phenolic and flavonid content while methanol extract was recorded to contain the lowest phenolic content. Of all extracts, the lowest amount of flavonoid was recorded in chloroform leaf extract which amounted to (28.21±0.63) mg quercetin equivalents/g of dried extract. The correlation between total phenolic and total antioxidant activity of fruit and leaf was studied using linear regression analysis, which is symbolized through Figures 1 and 2 respectively.

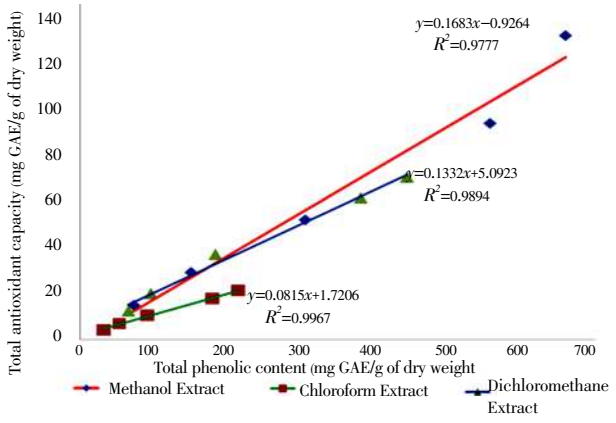

Figure 1. Correlation between total phenolic content and total antioxidant capacity of different extracts of fruit of *S. dulcis*.

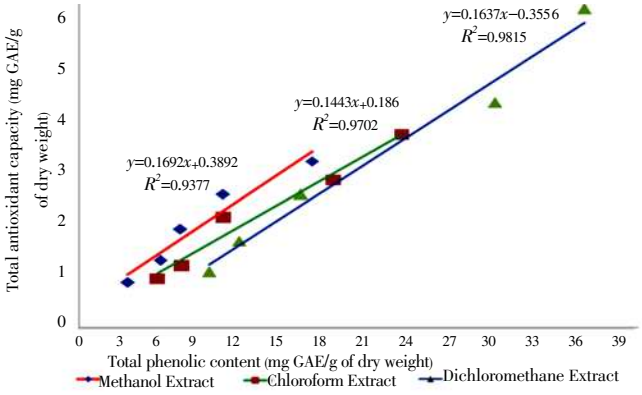

Figure 2. Correlation between total phenolic content and total antioxidant capacity of different extracts of leaf of *S. dulcis*.

3.3. DPPH free radical scavenging activity

The percentage of DPPH neutralization of all extracts was found to be concentration dependent. Methanol fraction of fruit produced the maximum free radical scavenging activity with IC_{50} value of 1.91 μg/mL, which was lower than that of reference antioxidant of the test. Among all leaf extract, dichloromethane fraction of leaf was found to produce the maximum free radical scavenging activity with IC_{50} value slightly higher than the standard antioxidant used in the test. In both fruit and leaf, chloroform fraction showed the weakest free radical scavenging activity with an IC_{50} value of 7.30 and 8.96 μg/mL for the fruit and leaf, respectively (Table 3). Figure 3 represents the percentage of DPPH neutralization activity of all extracts considered in the study.

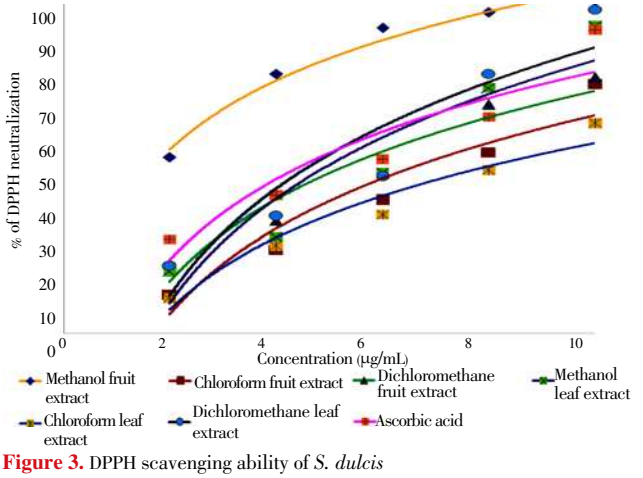

Figure 3. DPPH scavenging ability of *S. dulcis*

Table 3

DPPH free radical scavenging and reducing activity of the different extracts of *S. dulcis*.

Sample	IC_{50} (μg/mL)	EC_{50} (μg/mL)
Methanol fruit extract	1.91	3.58
Chloroform fruit extract	7.30	20.23
Dichloromethane fruit extract	5.85	8.45
Methanol leaf extract	5.37	12.45
Chloroform leaf extract	8.96	32.95
Dichloromethane leaf extract	5.00	9.37
Ascorbic acid	4.94	5.10

3.4. Reducing power assay

The reducing power of all extracts was also found to be concentration dependent. The EC_{50} value is described in Table 3 while the percentage of reducing power is illustrated in Figure 4. Like percentage of DPPH neutralization activity, methanol fruit extract provided the most intense reducing power with EC_{50} value of 3.58 μg/mL, which was lower than that of standard antioxidant used in the test. Among leaf extracts, dichloromethane fraction produced the most pronounced reducing power with an EC_{50} value of 9.37 μg/mL. The least reducing power was recorded in chloroform leaf extract with a comparatively higher EC_{50} value of 32.95 μg/mL.

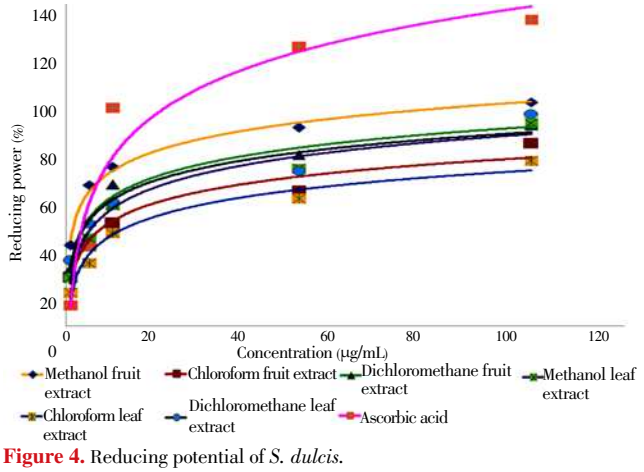

Figure 4. Reducing potential of *S. dulcis*.

3.5. Total antioxidant capacity

Total antioxidant capacity of all extracts is expressed in Table 2. The most powerful antioxidant activity was recorded in methanol fruit extract which amounted to (118.46±0.48) mg GAE equivalents/g of dry weight while the methanol leaf extract was found to provide the least antioxidant activity which amounted to (2.98±0.70) mg GAE equivalents/g of dry weight. In brief, the fruit extract showed stronger antioxidant capacity than the leaf extract. The higher correlations between total phenolic content and total antioxidant activity were detected for all extracts of fruit (R^2 range: 0.977 7–0.996 7) and leaves (R^2 range: 0.937 7– 0.981 5) of *S. dulcis*.

3.6. Antimicrobial activity

The results of different extracts of *S. dulcis* with disc diffusion method are shown in Table 4. The antimicrobial activity of all test fractions was tested using two different concentration *i.e.* 400 and 800 μg/disc. The antimicrobial activity increased with increasing concentration. Among fruit extract, the zones of inhibition at concentration of 400 and 800 μg/disc were ranged from 0.0 to 12.0 mm and 0.0 to 16.0 mm, respectively. Of leaf extract, the zones of inhibition at concentration of 400 and 800 μg/disc were ranged from 0.0 to 12.0 mm and 0.0 to 15.0 mm, respectively. All extracts showed variable inhibitory activity against the strains tested in the study while methanol fruit extract and dichloromethane leaf extract showed no activity against *Sacharomyces cerevacae*. Among the Gram-positive strains, the most pronounced inhibitory activity was found for methanol fruit extract against *Sarcina lutea* with the inhibition zone of 12 mm at 400 μg/disc and 16 mm at 800 μg/disc while methanol leaf extract showed potent inhibitory activity against *Bacillus subtilis* with the inhibition zone of 12 mm at 400 μg/disc and 16 mm at 800 μg/disc. Among the Gram-negative strains, the most potent inhibitory activity was found for chloroform fruit extract against *Shigella boydii* and *Salmonella paratyphi*. Methanol extract of both fruit and leaf showed equal inhibitory activity against *Pseudomonas aeruginosa* and the inhibition zone was 12 mm at 400 μg/disc and 15 mm at 800 μg/disc. The most potent antifungal activity was given by chloroform leaf extract against *Candida albicans*. Furthermore, chloroform leaf extract showed good antimicrobial activity against *Shigella dysentery*.

Table 4
Antimicrobial activity of the different extracts of *S. dulcis* (mm).

Strains	*S. dulcis* fruit (mg)						*S. dulcis* leaf (mg)						Control	
	Methanol		Chloroform		Dichloromethane		Methanol		Chloroform		Dichloromethane		Positive	Negative
	400	800	400	800	400	800	400	800	400	800	400	800		
B. cereus	5	9	7	10	5	8	7	9	8	11	6	9	25	0
B. subtilis	7	12	11	14	6	10	12	15	5	8	5	7	26	0
S. lutea	12	16	6	11	5	11	10	13	6	9	6	8	21	0
S. aureus	10	15	7	10	8	12	6	10	9	13	5	8	17	0
E. coli	7	11	5	8	7	10	6	9	7	10	9	11	27	0
S. paratyphi	10	15	12	15	9	12	9	11	8	11	6	10	25	0
S. typhi	9	13	6	9	10	14	10	14	8	12	5	8	20	0
S. boydii	11	15	12	16	8	11	7	11	6	9	9	13	23	0
S. dysentery	7	13	9	13	6	9	10	13	11	15	5	9	22	0
V. mimicus	6	10	7	11	5	9	8	11	7	10	9	11	22	0
P. aeruginosa	12	15	8	12	7	11	12	15	10	14	8	12	25	0
C. albicans	7	13	6	10	5	8	6	10	10	13	0	4	27	0
S. cerevacae	0	0	5	7	0	4	5	7	4	6	0	0	20	0

Table 5
Cytotoxic activity of the different extracts of *S. dulcis*

Sample	Probit at different concentration tested (μg/mL)										LC_{50} (μg/mL)	LC_{90} (μg/mL)	95% confidence interval
	0.781	1.563	3.125	6.25	12.5	25	50	100	200	400			
Methanol fruit extract	4.72	4.85	4.85	5.15	5.28	5.28	5.58	5.77	6.23	---	3.745	629.292	4.996-5.605
Chloroform fruit extract	4.23	4.56	4.56	4.85	4.85	5.00	5.44	5.44	5.77	6.23	12.778	1 109.913	4.710-5.475
Dichloromethane fruit extract	4.05	4.56	4.85	5.00	5.15	5.28	5.28	5.77	5.95	---	8.964	662.878	4.738-5.459
Methanol leaf extract	4.85	5.15	5.28	5.28	5.58	5.77	6.23	6.23	---	---	1.335	111.860	5.234-5.857
Chloroform leaf extract	4.23	4.56	4.56	4.72	4.85	5.15	5.44	5.44	5.77	---	14.057	1 784.177	4.653-5.284
Dichloromethane leaf extract	4.42	4.56	4.85	5.28	5.77	5.77	5.95	---	---	---	3.572	81.936	4.840-5.616
Vincristine sulfate	4.16	4.75	5.00	5.25	5.84	5.84	6.28	---	---	---	3.339	47.203	4.847-5.758

3.7. Cytotoxicity

The cytotoxic potential of all six extracts was compared with vincristine sulfate, the positive control which is illustrated in Table 5. Furthermore, the LC_{50}, LC_{90} and 95% confidence interval are also expressed in Table 5. The LC_{50} value for all extracts with 24 h observation was far lower than the cut off value for cytotoxicity. Methanolic leaf extract exhibited the lowest LC_{50} value (1.335 μg/mL) while chloroform leaf extract showed the highest LC_{50} value (14.057 μg/mL) among the other extracts. The LC_{50} value of vincristine sulfate was 3.339 μg/mL.

3.8. Thrombolytic activity

Thrombolytic activity of all the fruit and leaf extracts of *S. dulcis* is presented in Table 6. The maximum activity was recorded in chloroform fruit extract which amounted to (25.303±0.213)% of clot lysis while streptokinase exhibited a clot lysis of (50.005±0.277)%. Among leaf extracts, dichloromethane fraction showed maximum activity. The mean difference in clot lysis percentage between positive and negative control (sterile distilled water) was found statistically significant. Nevertheless, all extracts demonstrated statistically significant thrombolytic activity (P<0.001).

Table 6
Thrombolytic activity of the different extracts of *S. dulcis*.

Sample	Percentage of clot lysis
Methanol fruit extract	13.876±0.283
Chloroform fruit extract	25.303±0.213
Dichloromethane fruit extract	7.841±0.067
Methanol leaf extract	7.758±0.210
Chloroform leaf extract	6.486±0.210
Dichloromethane leaf extract	10.570±0.079
Streptokinase (positive control)	50.005±0.277
Water (blank)	2.610±0.360

4. Discussion

Both edible and non edible plants hold ample phenolic compounds which have the potential to exhibit multiple biological effects including antioxidant activity[36]. The hydroxyl groups of phenolic compounds allow them to exert direct antioxidative activity and were found to play a vital role in stabilizing lipid peroxidation[41,42]. They are known as powerful chain breaking antioxidants. However, flavonoid found in plants have antioxidant activity *in vitro* or *in vivo*[43]. Antioxidative potential of flavonoid depends on their chemical structure, especially of 3′,4′- orthodihydroxy group on the B cycle and the 4- carbonyl group on the C cycle. The 3-OH and 5-OH group on the C cycle are also relevant to the antioxidant activity. The hydroxyl group located on the C_3 of the C cycle of flavonols is recommended as the vital site for scavenging free radicals[44,45]. In general, the fruit extracts were found to have higher amount of phenolic and flavonoid components compared with leaf extracts.

The scavenging potential of all six extracts of *S. dulcis* was appraised through investigating their DPPH reduction against the positive control (L-ascorbic acid). DPPH radical loses its chromophore upon receiving proton from hydrogen donor. Consequently, increased concentration of phenolic compounds or number of hydroxyl group on aromatic ring boosts DPPH radical scavenging activity[46,47]. The antioxidative as well as the scavenging potential of extract is directly proportional to the DPPH reduction. The more antioxidants found in extract, the more DPPH reduction will occur. Higher DPPH reduction is associated with greater scavenging potential. Since all extracts showed dose dependent DPPH scavenging activity, these extracts may exert more pronounced and significant free radical scavenging activity. The antioxidant potential of the extracts measured by DPPH scavenging method was also expressed as 50% inhibitory concentration, IC_{50} values. Methanol fruit extract was found to have the lowest IC_{50} value among the other extracts. The result of our study indicates a strong relationship between phenolic content and DPPH scavenging as well as antioxidant activities, suggesting that the phenolic compounds are probably responsible for the antioxidant activity of *S. dulcis*.

The reducing potential of a compound or plant may serve as a remarkable indicator of its potential antioxidant activity. According to the report of Seddik *et al.*, reducing potential of tannins inhibits the formation of liver peroxides, thereby prevents liver injury[48]. Like DPPH scavenging activity, the reducing potential of the sample was found to be dose dependent. Higher dose exerts greater reducing

potential. The antioxidant potential of the extracts measured by reducing assay was also expressed as 50% effective concentration, EC_{50} values. Like DPPH scavenging activity, methanol fruit extract was found to have the lowest EC_{50} value among the other extracts.

The results of total antioxidant capacity were correlated with the phenolic contents. Methanol fruit extract was found to exert the greatest total antioxidant capacity which harmonized the results of total phenolic content of our study. The results of our study are also consistent with the outcomes of many research groups who reported positive correlation between total phenolic content and antioxidant activity[49-51]. In our test, all extracts showed a higher correlation between phenolic content and antioxidant activity with a R^2 range of 0.937 7-0.996 7. According to the report of Ronald *et al.*, antioxidant activity in DPPH and phosphomolybdenum assay may be exerted through either hydrogen atom transfer or single electron transfer mechanism[52].

Since plants are rich in various types of secondary metabolites including tannins, terpenoids, alkaloids and flavonoid, they have been found to exert *in vitro* antimicrobial property[53]. In our study, antimicrobial activity of various fruit and leaf extracts of *S. dulcis* is evaluated by disc diffusion method. Two different doses of extracts were used in the test and compared with the positive control. In our study, methanol fruit extract and dichloromethane leaf extract don't exhibit any activity against *S. cerevacae*. All extracts showed dose dependent activity. Nevertheless, the inhibition zone produced by the commercially available positive control was larger than those produced by the extracts. The presence of very minute concentrations of bioactive compounds in the plant extract may contribute to the poor antimicrobial activity[54]. However, the plant extracts were found to be effective against both bacteria (Gram-positive and Gram-negative) and fungi. This sort of activity gives an indication of the presence of antimicrobial compounds with broad spectrum or simply general metabolic toxins[55]. Therefore, extracts of *S. dulcis* may be helpful in finding antimicrobial compounds.

Since any compound or extract can exert antimicrobial and antioxidant activity as a result of its toxic effects on the cells, determination of the toxic effect of antimicrobial and antioxidant agents on host cell is mandatory[56]. For this purpose, brine shrimp lethality test was performed. The results observed in 24 h were found to be dose dependent for all extracts. Any extract or pure compound is considered as a potential cytotoxic and toxic substance only if LC_{50} is less than 1 000 μg/mL[57,58].

Thrombolytic activity of two different parts (the fruit and leaf) of *S. dulcis* was measured and compared with streptokinase (the positive control) and sterile distilled water (the negative control). In the study, chloroform fruit extract was found to provide the maximum thrombolytic activity. However, the percentage of clot lysis produced by the commercially available positive control was far greater than those produced by the extracts. The presence of minor amount of bioactive thrombolytic compound in the extracts may contribute to the weak result. All extracts exhibited statistically significant thrombolytic activity ($P<0.001$).

In conclusion, the current study illustrates the fruit and leaf of *S. dulcis* should be regarded as a valuable source of material for human health, as an antioxidant, antimicrobial, cytotoxic and thrombolytic agent. Further studies are desirable to characterize and isolate the unknown underlying components in order to establish their pharmacological properties which could provide valuable lead compounds in the respective therapeutic area.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

In spite of the great advances observed in modern medicine, plants still make an important contribution to health care. Due to the adverse effects of synthetic agents, search for alternative herbal medicine is required. Thus, emphasis is now given on the screening of biological activities of medicinal plants.

Research frontiers

The present study demonstrates potential antioxidant, antimicrobial, cytotoxic and thrombolytic activities of fruit and leaves extracts of *S. dulcis* and assessed by different biochemical parameters, disc diffusion methods, brine shrimp lethality bioassay and *in vitro* clot lysis model.

Related reports

DPPH radicals have been widely used to evaluate antioxidant properties. Brine shrimp lethality bioassay is a rapid bioassay indicating cytotoxicity while clot lysis model is a simple technique evaluating thrombolytic activity. Medicinal plants, rich in bioactive compounds including phenolics, terpenoids, alkaloids and flavonoid, have been found to possess antioxidant, cytotoxic, antimicrobial property.

Innovations and breakthroughs

S. dulcis is used in eye infections, itchiness, internal ulceration, sore throat and inflammation. In the present research, authors have demonstrated the antioxidant, antimicrobial, cytotoxic and thrombolytic potential of various fruit and leaves extracts of *S. dulcis* which could provide valuable lead compounds in the respective therapeutic area.

Applications

The results of the study illustrate that fruit and leaf of *S. dulcis* should be regarded as a valuable source of material for human health, as an antioxidant, antimicrobial, cytotoxic and thrombolytic agent. The results justify the traditional use of the plant in the treatment of infectious disease.

Peer review

This is a good study in which authors investigated the antioxidant, antimicrobial, cytotoxic and thrombolytic property of the fruits and leaves of *S. dulcis* using different *in vitro* biochemical analysis, disc diffusion methods and *in vivo* bioassay *etc*. The methanolic fruit extracts of the plant exhibited the most promising results and might be a potential source of lead compounds for aforementioned bioactivities.

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