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Short communication

Antioxidant and free radical scavenging potential of *Citrullus colocynthis* (L.) Schrad. methanolic fruit extract

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Citrullus colocynthis (L.) Schrad. (*Cucurbitaceae*) is a medicinal plant traditionally used as an abortifacient and to treat constipation, oedema, bacterial infections, cancer and diabetes. Preliminary phytochemical screening of the plant showed the presence of large amounts of phenolics and flavonoids. Subsequent quantification showed the presence of 0.74% (*m/m*) phenolics (calculated as gallic acid) and 0.13% (*m/m*) flavonoids calculated as catechin equivalents per 100 g of fresh mass. The presence of phenolic compounds prompted us to evaluate its antioxidant activity. In the present study, methanolic fruit extract of *C. colocynthis* was screened to evaluate its free--radical scavenging effect. The highest antioxidant and free radical scavenging ability of the fruit extract was observed at a concentration of 2500 μg mL^{-1}.

Keywords: *Citrullus colocynthis* fruit, *Cucurbitaceae*, methanolic extract, antioxidants, free radical scavenging

Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl, nitric oxide radicals, play an important role in oxidative stress related to the pathogenesis of various important diseases (1, 2). Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. Antioxidant-based drugs/formulations for the prevention and treatment of complex diseases, like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer, have appeared in the last three decades (3). This has attracted a great deal of research interest in natural antioxidants. Flavonoids and phenolic compounds are widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic, *etc*. (4). The aim of the present investigation was to evaluate *in vitro* antioxidant and free radical scavenging activity of the *Citrullus colocynthis* fruit extract. The fruits of *Citrullus colocynthis*, commonly known as bitter apple, are bitter, acrid, cooling, cathartic, carminative, antipyretic, anthelmintic and are useful in hypoglycemia, tumors, ascites, leucoderma, ulcers, asthma, bronchitis, urethorrhea, jaundice, dyspepsia, constipation, elephantiasis and splenomegaly (5). Fruit extract exhibits nematicidal properties.

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MATERIALS AND METHODS

Chemicals

Chemicals used in this study were 1,1-diphenyl-2-picrylhydrazyl (DPPH) obtained from Sigma-Aldrich, India, NADH and sulfanilamide obtained from Himedia, Laboratories Pvt. Ltd., India, Folin-Ciocalteu reagent, potassium ferricyanide and sodium nitroprusside obtained from Qualigens Fine Chemicals, Glaxo Smithkline Pharmaceutical Ltd., India, naphthylethylenediamine dihydrochloride, *N*-1-naphthylethylenediamine dihydrochloride, sodium nitrite, trichloroacetic acid, butylated hydroxy anisole (BHA), ascorbic acid, α-tocopheryl acetate, ethylenediamine tetraacetic acid, phosphoric acid, nitro blue tetrazolium, phenazine methosulfate, ferrous ammonium sulfate, DMSO are obtained from Sd Fine Chemicals Ltd, India. All reagents used in the study were of analytical grade.

Plant material

Citrullus colocynthis (*Cucurbitaceae*) fruits were collected from the areas near the Rewari district, Haryana, India, during October, 2006 and authenticated at the Department of Botany, Kurukshetra University, Kurukshetra, Haryana, India. The fruits were cleaned and dried in the shade, then powdered to 0.422 mm mesh size and stored in an airtight container at 25 °C.

Extraction

Citrullus colocynthis fruits (100 g) in powdered form were extracted with methanol using a Soxhlet assembly for 48 h, filtered and last traces of the solvent were evaporated under reduced pressure in a rotary evaporator. The yield was 2.78 g of dry extract.

Total phenolic content

The total phenolic content of *Citrullus colocynthis* fruit (CCF) extract was determined spectrometrically (6). Folin-Ciocalteu's reagent, 1 mL previously diluted with 20 mL distilled water, was added to 1 mL of sample (250 μg mL^{-1}) and mixed thoroughly. To the mixture, 4 mL of sodium carbonate (75 g L^{-1}) and 10 mL of water were added and mixed well. The mixture was allowed to stand for 2 h at room temperature. Contents were then centrifuged at 2000 × *g* for 5 min and the absorbance of the supernatant was taken at 760 nm using a double beam spectrophotometer 2202 (Systronics, India).

A standard curve was obtained using various concentrations of gallic acid. Results were expressed as percentage of gallic acid equivalents (GAE) per 100 g fresh mass.

Total flavonoid assay

Total flavonoid contents were measured with the aluminum chloride colorimetric assay (7). Methanolic fruit extracts or standard solution of catechin (500 μg mL^{-1}) was added to 10 mL volumetric flask containing 4 mL of water. To the above mixture, 0.3 mL of 5% $NaNO_2$ was added. After 5 minutes, 0.3 mL of 10% $AlCl_3$ was added. After 6 min,

2 mL of 1 mol L^{-1} NaOH was added and the total volume was made up to 10 mL with water. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm. Total flavonoid content of the fruit was expressed as percentage of catechin equivalent per 100 g fresh mass.

DPPH free radical scavenging activity

The free-radical scavenging activity of CCF extract was measured by the decrease in absorbance of methanolic solution of DPPH (8). A stock solution of DPPH (33 mg L^{-1}) was prepared in methanol and 5 mL of this stock solution was added to 1 mL of the CCF extract solution at different concentrations (250, 500, 1000, 1500, 2000 and 2500 μg mL^{-1}). After 30 min, absorbance was measured at 517 nm and compared with the standards, *i.e.*, ascorbic acid, BHA and α-tocopherol (10–50 μg mL^{-1}). Scavenging activity was expressed as the percentage inhibition.

Hydroxyl radical scavenging activity

Methanolic extract at different concentrations was placed in a test tube and evaporated to dryness. One mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of 0.018% EDTA, 1 mL of DMSO [0.85%, *V/V*, in 0.1 mol L^{-1} phosphate buffer, pH 7.4] and 0.5 mL of 0.22% ascorbic acid were added to each tube (9). The tubes were capped tightly and heated in a water bath at 80–90 °C for 15 min. The reaction was terminated by adding 1 mL of ice-cold TCA (17.5% *m/V*). Three ml of Nash reagent (75.0 g ammonium acetate, 3 mL glacial acetic acid and 2 mL acetyl acetone were mixed and water was added to a total volume of 1 L) was added to each tube; the tubes were left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured at 412 nm against a blank of the reagent. Percentage inhibition was determined by comparing the results of the test and standard compounds.

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (40 mmol L^{-1}) was prepared in phosphate buffer (pH 7.4). Different concentrations (250–2500 μg mL^{-1}) of CCF were added to the hydrogen peroxide solution (40 mmol L^{-1}, 0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide (10). Percentage scavenging of hydrogen peroxide of the CCF extract and standard compounds was calculated.

Superoxide radical scavenging assay

The reaction mixture consisting of 1 mL of nitro blue tetrazolium (NBT) solution (156 mmol L^{-1} NBT in phosphate buffer, pH 7.4), 1 mL NADH solution (468 mmol L^{-1} NADH in phosphate buffer, pH 7.4), and 1 mL of sample solution of CCF extract was mixed. The reaction was started by adding 100 mL of phenazine methosulfate (PMS) solution (60 mmol L^{-1} PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance was measured at 560 nm

against blank sample and compared with the standards (10, 11). Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured spectrophotometrically (12). Sodium nitroprusside (5 mmol L^{-1}) in phosphate buffered saline pH 7.4, was mixed with different concentrations of the extract (250–2500 μg mL^{-1}) prepared in methanol and incubated at 25 °C for 30 min. A control without the test compound, but with an equivalent amount of methanol, was taken. After 30 min, 1.5 mL of the incubated solution was removed and diluted with 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with *N*-1-naphthylethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard.

Statistical analysis

Values were represented as mean ± SD of three parallel measurements and data were analyzed using the *t*-test.

RESULTS AND DISCUSSION

From the results on the total phenolic content, it was found that there was 0.74% of gallic acid equivalents of phenolic compounds while the total flavonoid content was 0.13% of catechin equivalent of fresh mass of *C. colocynthis* fruit extract. The results of antioxidant and free radical scavenging activity are given in Table I.

The free radical scavenging activity was evaluated by using various *in vitro* assays. DPPH radical was used as a substrate to evaluate the free radical scavenging activity of CCF extract. The scavenging effect of CCF extract on the DPPH radical was 88.0 ± 2.7% ($p < 0.005$), at a concentration of 2500 μg mL^{-1} compared to the scavenging effects of ascorbic acid, BHA and α-tocopherol at 50 μg mL^{-1} of 89.5 ± 1.1, 83.2 ± 1.1 and 67.5 ± 0.8% ($p < 0.05$) respectively.

Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and enormous biological damage (13). The percentage of hydroxyl radical scavenging increased with the increasing concentration of fruit extract. The percentage of H_2O_2 scavenging activity of CCF was found to be 62.7 ± 3.5 ($p < 0.001$) at 2500 μg mL^{-1}, and antioxidant activity of BHA and α-tocopherol was 89.3 ± 3.1 % ($p < 0.05$) and and 94.5 ± 2.5 % ($p < 0.05$), respectively at a concentration of 50 μg mL^{-1}. H_2O_2 itself is not very reactive, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells. Thus, removal of H_2O_2 is very important for protection of food systems.

The superoxide anion radical scavenging activity of CCF was assayed using the PMS-NADH system. The percentage inhibition of superoxide generation by CCF at 2500

μg mL^{-1} concentration was found to be 71.3 ± 3.2% ($p < 0.005$). On the other hand, ascorbic acid, BHA and α-tocopherol at 50 μg mL^{-1} exerted 85.6 ± 2.5, 68.0 ± 4.6 and 74.5 ± 2.7% ($p < 0.05$) inhibition of the superoxide radical.

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and

Table I. Antioxidant profile of the Citrullus colocynthis *fruit extract*[a]

Sample	Conc. (μg mL^{-1})	DPPH radical scavenging (%)	Hydroxyl radical scavenging (%)	Hydrogen peroxide scavenging (%)	Superoxide anion scavenging (%)	Nitric oxide scavenging (%)
CCF	(2500)	88.0 ± 2.7	71.4 ± 3.2	62.7 ± 3.5	71.3 ± 3.2	61.4 ± 3.8
Ascorbic acid	(50)	89.5 ± 1.1	–	–	85.7 ± 2.5	86.0 ± 3.5
BHA	(50)	83.2 ± 1.1	83.2 ± 3.2	89.3 ± 3.1	68.0 ± 4.6	
α-Tocopherol	(50)	67.5 ± 0.8	–	94.5 ± 2.5	74.5 ± 2.7	

[a] Mean ± SD, $n = 3$.

regulation of cell mediated toxicity (14). The percentage inhibition of nitric oxide generation by CCF at 2500 μg mL^{-1} concentration was found to be 61.4 ± 3.8% ($p < 0.005$). On the other hand, ascorbic acid at 50 μg mL^{-1} concentration showed 86.0 ± 3.5% ($p < 0.05$) inhibition of nitric oxide.

CONCLUSIONS

Free radical scavenging effect of CCF increases with increasing concentration and maximum antioxidant activity was observed at 2500 μg mL^{-1}. Antioxidant activity may be due to phenolic compounds in CCF but further work should be done on the isolation and identification of other antioxidant components of *Citrullus colocynthis*.

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S A Ž E T A K

Antioksidativni potencijal i sposobnost hvatanja slobodnih radikala metanolnog ekstrakta plodova *Citrullus colocynthis* (L.) Schrad.

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Citrullus colocynthis (L.) Schrad. (*Cucurbitaceae*) je ljekovita biljka koja se tradicionalno upotrebljava kao abortiv i za liječenje konstipacije, edema, bakterijskih infekcija, karcinoma i dijabetesa. Preliminarno fitokemijsko pretraživanje ukazalo je na prisutnost velikih količina fenola i flavonoida. Udio fenola bio je 0,74% (preračunato na galnu kiselinu), a flavonoida 0,13% preračunato na ekvivalente katehina na 100 g svježe mase. Zbog prisutnosti fenolnih spojeva ispitivano je antioksidativno djelovanje i sposobnost hvatanja slobodnih radikala metanolnog ekstrakta plodova. Koncentracija 2500 μg mL^{-1} imala je najjači učinak.

Ključne riječi: plod biljke *Citrullus colocynthis (Cucurbitaceae)*, metanolni ekstrakt, antioksidansi, hvatanje slobodnih radikala

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