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In vitro antioxidant activity and total phenolic content of *Citrullus colocynthis* fruits

***S. Saini**

Department of Botany, Janta Vidya Mandir Ganpat Rai Rasiwasia College, Charkhi Dadri-127306, Haryana, India

***Corresponding Author: Dr. Sushila Saini**
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ABSTRACT

Generation of free radicals is the cause of oxidative stress related diseases and antioxidants by quenching free radicals have a significant contribution in the prevention of diseases. As synthetic antioxidants are responsible for causing toxicity, mutagenesis and tumour, search for antioxidants of plant origin is becoming more prominent. Present study was carried out to determine total phenolic content, reducing power, metal chelating and free radical (DPPH, superoxide and hydroxyl) scavenging activity of fruit extract of *Citrullus colocynthis*. Total phenolic content was determined using the Folin-ciocalteu reagent and expressed as mg/g gallic acid equivalent. Antioxidant activity was found to be highest in methanolic extract which also possesses highest phenolic content (57.34mg/g). A Positive correlation was observed between total phenolic content and different antioxidant assays thus confirming the role of phenolics in contributing to the antioxidant potential of *C. colocynthis* fruits

Keywords: *Citrullus colocynthis*, total phenolic content, DPPH radical, superoxide radicle, oxidative stress

INTRODUCTION

Antioxidants are secondary metabolites having redox properties that neutralize free radicals by donating an electron thus terminating the chain reactions. Free radicals are generated in the biological system by various endogenous (oxidation reactions) or exogenous (UV rays, pollutants, ionizing radiation) agencies. Under normal conditions a balance is maintained between generation of free radicals and endogenous antioxidant system. Imbalance in the system causes overproduction of free radicals initiating oxidation of proteins, lipids, carbohydrates and nucleic acids resulting in membrane damage, DNA breakage and eventually cell death. Oxidative stress induced by free radicals can result in cancer, heart diseases, neurological disorder, arteriosclerosis, and aging [1,2]. Synthetic oxidants such as BHA (Butylated hydroxyanisole) and BHT (Butylated hydroxytoluene) have good antioxidant properties but their use is limited due to toxicity and mutagenesis leading to cancer [3]. In this context search for novel antioxidants from plant sources is still in great demand as they are safe, easily available and economical. In India local herbs and other plants have been a source of treating various diseases for a long time. In the Ayurvedic system of medicine several plants have been extensively used for the treatment of various chronic diseases, some of which show great antioxidant potential. Bioactive compounds such as flavonoids and other polyphenolic compounds reported from root, leaves, stem and bark of plants are potent free radical scavengers [4,5,6].

Bitter apple also called *Citrullus colocynthis* (L.) Schard. belonging to the family Cucurbitaceae is a well-recognized plant in traditional systems of medicine in different parts of the world. It is an annual or perennial viny herb that grows well in barren areas with sandy soil. Its fruits are large ovoid, intensely bitter, pungent and are used in the treatment of constipation, enlarged spleen, fever, edema, diabetes and bacterial infections. Phytochemical analysis revealed the presence of alkaloids, flavonoids, saponins, triterpenoids, steroids, cucurbitacin A, B, C, D, E and coumarin glycosides from different parts of *C. colocynthis* [7,8]. As considerable interest has been generated in the role of antioxidants in oxidative stress related diseases therefore the present study was planned to evaluate the free radical scavenging activity and total phenolic content of different extracts of *C. colocynthis* fruits.

MATERIALS AND METHODS

Preparation of different extracts

Plants of *Citrullus* were collected from rural areas of Southern Haryana and authenticated at FRI, Dehradun with the voucher number 10814. After collection fruits were dried

in shade and pulverized to find powder using a grinder. 100 gram of the powdered material was extracted with five different solvents (petroleum ether, benzene, chloroform, methanol, and water) filtered, evaporated under vacuum on a rotary evaporator and stored at 40^0C for further use. To assess total phenolic content and antioxidant activity varying concentration (from 0.2mg/ml to 1 mg/ml) of extracts were used.

Assessment of total phenolic content and antioxidant activity

Total phenolic content (TPC) was determined by the method of Singleton and Rossi [9] with slight modifications and expressed as mg/g gallic acid equivalent (GAE). DPPH free radical scavenging activity of different extracts was detected by their ability to bleach DPPH radical according to the procedure adopted by Lee et al [10] with ascorbic acid (10 µg/ml-50 µg/ml) used as standard. Ability of the extract to scavenge superoxide radical was assayed on the basis of reduction of Nitro blue tetrazolium according to Liu et al [11] and compared with standard BHT. To measure the ability of extract for scavenging hydroxyl radical generated in the Fenton reaction, a method proposed by Kunchandy and Rao [12] was employed. Chelation of metal ions and inhibition of Ferrozine ferrous complex formation by extract was studied by method of Dinis et al [13]. Reductive potential of extracts was assayed as described previously by Yen and Duh [14] and compared with ascorbic acid as standard. Increased absorbance at 700 nm establishes higher reducing power of test samples.

All the tests were done in triplicate and percentage inhibition was calculated as follows.

% Inhibition = A (control) - A (sample or standard) / A (control) × 100,

where A (control) = absorbance of the control; A (sample or standard) = absorbance of sample extract or standard

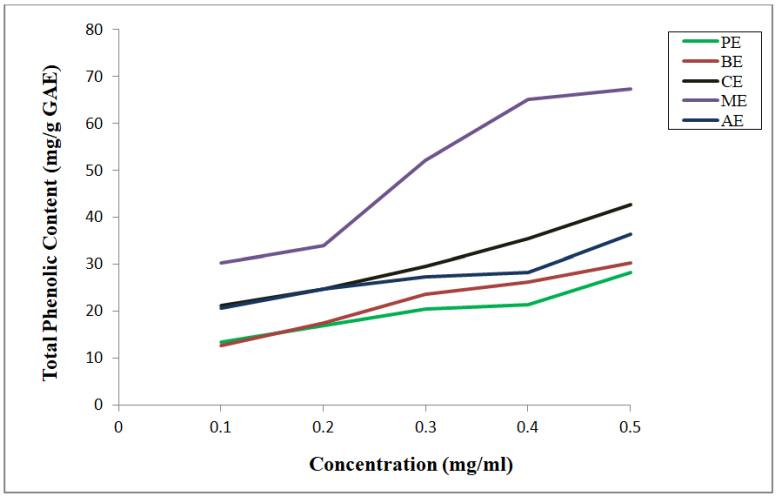
STATISTICAL ANALYSIS

On way ANOVA (Analysis of Variance) followed by Duncan's multiple range tests at $p< 0.05$ level of significance were used. IC_{50} value for extracts and standard was calculated and Pearson correlation coefficient was used to estimate correlation between TPC and various antioxidant assays.

RESULTS AND DISCUSSION

Total phenolic content (TPC)

Plant phenolics by acting as hydrogen or electron donor can stabilize unpaired electrons of free radicals or can chelate the trace elements. These phenolics compounds are responsible for antioxidant activity of plants thereby preventing the free radical induced oxidative stress [15]. Usually extracts showing high antioxidant activity generally have high phenolic content. Methanolic extract of *C. colocynthis* fruits have highest TPC (57mgGAE/g). Value of TPC in different fruit extracts decreased in the following order: methanolic> chloroform> aqueous> benzene> petroleum ether extracts (Figure 1).



(PE- petroleum ether extract, BE- benzene extract, CE-chloroform extract, ME- methanol extract, AE- aqueous extract).

Fig 1: Total phenolic content (TPC) of fruit extracts of *C. colocynthis*

DPPH radical scavenging activity

This assay is a simple, fast and most common way for evaluation of antioxidant activity of plant extracts. DPPH is a stable free radical and gives violet colour in alcoholic solution that fades to yellow when it is reduced to DPPH-H in presence of antioxidants [4,16]. Plant extracts along with the standard ascorbic acid depicted a concentration dependent increase in scavenging of DPPH free radicals. Highest scavenging ability was shown by methanolic extract followed by chloroform> benzene> aqueous> petroleum ether extracts (Table 1). IC_{50} values of all the extracts were high as compared to ascorbic acid indicating them to be moderate scavengers compared to standard.

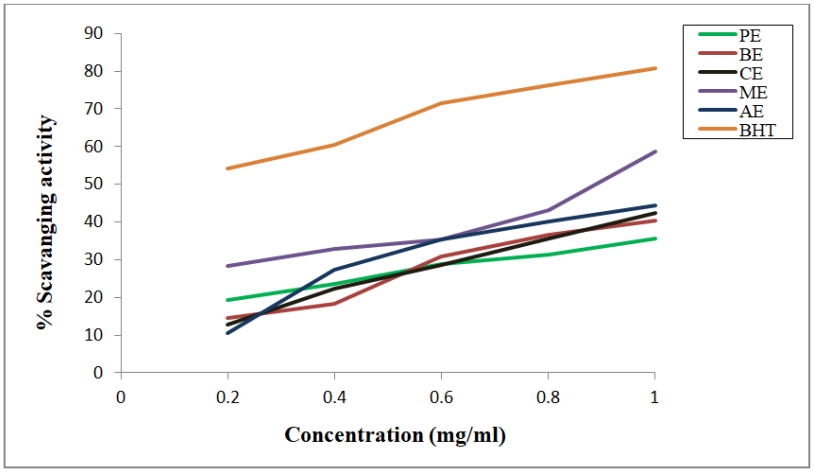
Table 1: DPPH free radical scavenging activity (%) of fruit extracts of *Citrullus colocynthis*

Fruit extracts							
Concentration (mg/ml)	**PE**	**BE**	**CE**	**ME**	**AE**	**Concentration (µg/ml) of AS**	**AS**
0.2	9.42±0.46^{e}	12.07±1.05^{e}	28.62±0.54^{e}	34.19±0.42^{e}	12.86±0.17^{e}	10	20.84±0.62^{e}
0.4	12.22±0.43^{d}	15.84±0.37^{d}	32.87±0.47^{d}	38.12±0.57^{d}	16.65±0.42^{d}	20	38.63±0.40^{d}
0.6	15.88±0.68^{c}	27.04±0.42^{c}	35.04±0.37^{c}	44.67±0.39^{c}	20.11±0.35^{c}	30	75.17±0.60^{c}
0.8	20.20±1.15^{b}	38.63±0.17^{b}	40.83±0.35^{b}	50.20±1.02^{b}	31.57±0.42^{b}	40	80.28±0.12^{b}
1.0	27.89±0.42^{a}	42.96±0.60^{a}	55.23±0.74^{a}	68.39±0.28^{a}	35.69±0.44^{a}	50	84.80±0.66^{a}

Values are expressed as mean±S.D., (n=3). Values with in the column not sharing common superscript letters (a-e) differ significantly at p<0.05 by Duncan's multiple range tests. PE- petroleum ether, BE- Benzene extract, CE- Chloroform extract, ME- Methanol extract, AE- Aqueous extract, AS- Ascorbic acid

Superoxide radical scavenging activity

During aerobic respiration generation of mildly reactive superoxide anion is a usual phenomenon. However in cells superoxide anion is the cause of generation of more potent reactive oxygen species such as hydrogen peroxide, singlet oxygen, and hydroxyl radical which can inactivate enzymes, breakdown DNA and degrade cell membranes leading to cell death [17]. Extracts and standard BHT (Butylated hydroxytoluene) depicted a concentration dependent decrease in absorbance at 560 nm establishing their ability to quench superoxide radicals in reaction mixture (Figure 2). Methanolic extract compared with other solvents exhibited maximum inhibition (58.76%) at 1.0 mg/ml concentration. The IC_{50} value for methanolic extract was 0.84 mg/ml and for standard BHT was 0.18 mg/ml.

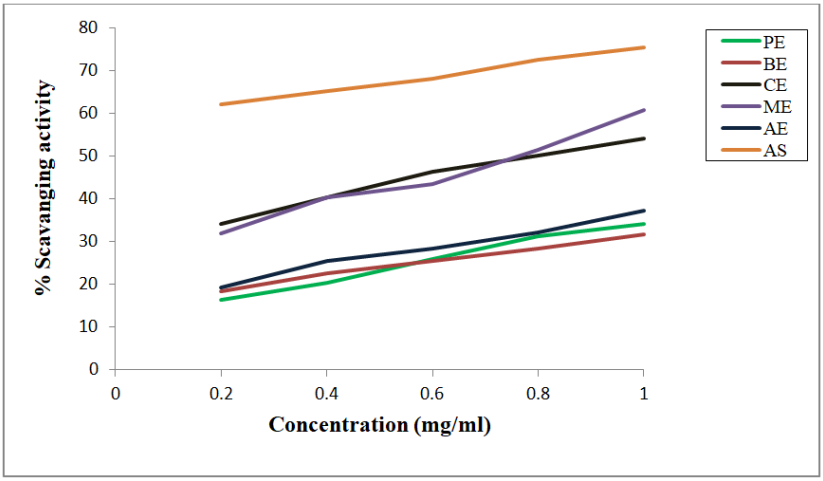


(PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, BHT- Butylated hydroxytoluene).

Fig 2: Superoxide radical scavenging activity (%) of fruit extracts of *C. colocynthis*

Hydroxyl radical scavenging activity

Hydroxyl radicals are one of the most potent reactive oxygen species having short life span and are capable of causing detrimental effects on biomolecules including proteins and nucleic acids. Due to highly reactive nature they cause enormous damage to biological system and to the individual as a whole. Hydroxyl radicals are generated from the hydrogen peroxide in the Haber-Weiss/ Fenton reaction in presence of ferrous ions [18,19]. Different extracts of *Citrullus colocynthis* prevented the generation of hydroxyl radical in a dose dependent manner. The percentage inhibition of hydroxyl radical in fruit extracts was maximum in methanolic extract followed by chloroform> aqueous> petroleum ether > benzene extracts (Figure 3). For methanolic extract percentage inhibition varies from 31.95% at 0.2mg/ml to 60.86% at 1.0 mg/ml. The IC_{50} value of methanolic extract was 0.78 mg/ml while that for standard ascorbic acid was 0.092 mg/ml.

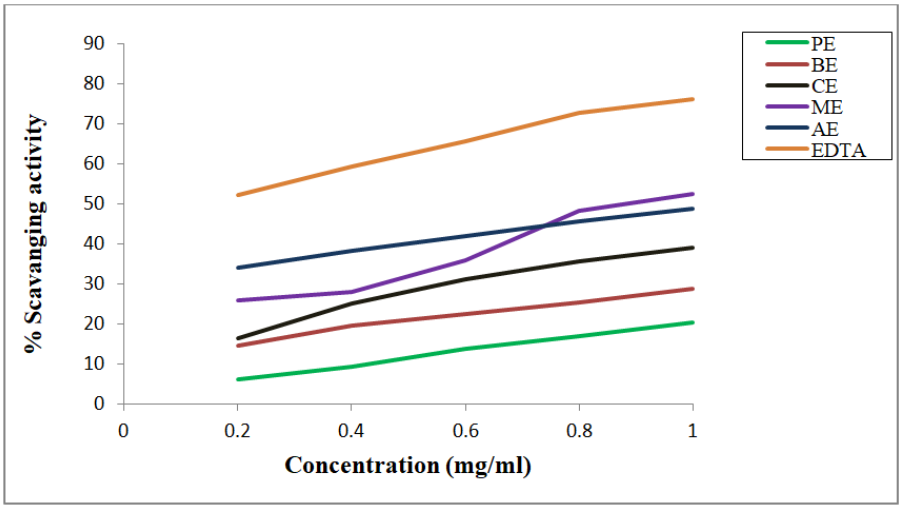


(PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, AS- Ascorbic acid).

Fig 3: Hydroxyl radical scavenging activity (%) of fruit extracts of *C. colocynthis*

Metal Chelating activity

Under inflammatory conditions there is an increased level of ferrous ions leading to oxidative damage. Chelation of ferrous ions leads to their mobilisation thus expelling them out of the body by urination or defecation. Plant extracts having antioxidants are good ferrous ion chelators thus minimising their concentration and inhibiting their capacity to generate free radicals hence protecting against oxidative stress [20,21]. In the present study *C. colocynthis* extracts possess remarkable efficacy to chelate free metal ions thus inhibiting iron-ferrozine complex formation leading to decreased absorbance at 560 nm as depicted in figure 4. Methanolic extract was the most effective in chelating metal ions followed by aqueous> chloroform> benzene> petroleum ether extracts. Methanolic extract (0.96 mg/ml) has much higher IC_{50} value than standard EDTA (0.19 mg/ml).

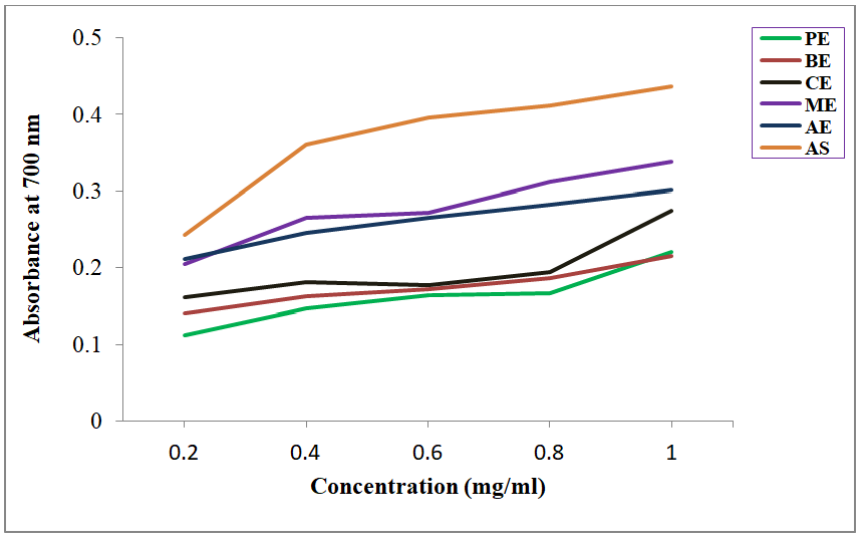


(PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, EDTA- Ethylenediamine tetra acetic acid).

Fig 4: Metal chelating activity (%) of fruit extracts of *C. colocynthis*

Reducing power assay

Reducing power assay was used to test the capability of plant extracts to donate electrons. In this assay, presence of reductones in test samples reduces ferric cyanide complex to ferrocyanide leading to production of various shades of green or blue depending on their reducing ability [22]. Increased absorbance with rising concentration of test samples indicates higher reduction ability. Analysis of different solvent showed that methanolic extract contains more reducing power ability as compared to other tested extracts but lower than standard ascorbic acid. The reductive potential of different extract ranked in the sequence: methanolic> aqueous> chloroform> petroleum ether> benzene extracts (Figure 5).



(PE- petroleum ether extract, BE- benzene extract, CE- chloroform extract, ME- methanol extract, AE- aqueous extract, AS- Ascorbic acid).

Fig 5: Reducing power assay of fruit extracts of *C. colocynthis*

Correlation of Total phenolic content and antioxidant activity

Phenolic compounds have antioxidant properties because their aromatic rings bearing one or more hydroxyl groups contribute as electron donors. Reports from literature survey exhibited the role of phenolics in quenching free radicals, as metal chelators and helping in peroxide decomposition thus preventing onset of oxidative stress related diseases [23,24]. Methanolic extract of *C. colocynthis* fruits showed significant positive correlation between total phenolic content and various antioxidant assays with value of correlation coefficient being 0.85805, 0.839101, 0.904303 and 0.946752 respectively for DPPH, superoxide, hydroxyl and metal chelating assays thus establishing the role of phenolic compounds towards its antioxidant activity (Table 2). Phytochemical analysis of various parts of *C. colocynthis* also revealed the presence of bioactive compounds [25,26]. Najafi et al [27] reported the presence of alkaloids, flavonoids, glycosides and saponosides from *C. colocynthis* leaves ethanolic and aqueous extracts. From hydromethanolic extract of *C. colocynthis* fruits three flavone glucosides such as isovitexin, isosaponarin and iso-orientin-3'-O-methyl ether and two cucurbitacin glucosides (2-O-β-D-glucopyranosylcucurbitacin and 2-O-β-D-glucopyranosylcucurbitacin) were isolated [28]. In another study, Sultan et al [29] reported the presence of 1.39mg flavonoids, 0.52mg saponosides, 1.64mg alkaloids, 1.64mg phenolic compounds and 30.12mg ascorbic acid per 100 mg of entire *C. colocynthis* plants. Thus the presences of these phytocompounds confer to the plant antioxidant activity.

Table 2: Correlation analysis between different antioxidant tests with their respective total phenolic content at 1 mg/ml concentration in *C. colocynthis* fruits methanolic extract

Assays	Total phenolics in fruits	
	r	R^2
DPPH radical scavenging	0.85805*	0.736*
Superoxide radical scavenging	0.839101*	0.704*
Hydroxyl radical scavenging	0.904303*	0.817*
Metal chelating assay	0.946752*	0.896*

r- correlation coefficient, R^2- coefficient of determination, *significance at p<0.05

CONCLUSION

The present findings validate the role of *C. colocynthis* as a therapeutic agent against oxidative stress. Antioxidant activity of the plant may be due to the presence of phenolic compounds that are able to donate electrons to neutralise the free radicals. To fully utilise its potential further investigation is needed to purify and characterize bioactive compounds.

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