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Evaluation of antioxidant activity of *Citrullus colocynthis* (L.) Schrad. extracts and their effect on urease activity

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Abstract

This study was aimed to evaluate the antioxidative and urease inhibitory properties of the extracts from *Citrullus colocynthis* L. The extracts of the root and pulp of fruit were prepared by soxhlet apparatus in methanol as solvent. The antioxidative activities, including DPPH radical scavenging activity, reducing power and total antioxidant activity were studied *in vitro*. The results showed that both tested extracts have antioxidative characteristics and it was found that the antioxidative activities of all the extracts increased with the increase of the concentration. In this report the methanolic extracts of *Citrullus colocynthis* root and pulp were evaluated for their effect on inhibition of soybean urease using the indophenol method as described by Weatherburn. The inhibition potency was measured by spectroscopy technique at 630 nm which attributes to released ammonium. The extracts showed inhibitory activities with IC_{50} 7.31µg/µl for root extract and 13.71µg/µl for pulp extract.

Introduction

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. 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 (Kumar & Kayastha., 2010). Approximately 50% of the earth's population is infected with *Helicobacter pylori* (Balasubramanian & Ponnuraj., 2010). It has been implicated in the etiology of chronic gastritis and peptic ulcer, in both adults and children. The World Health Organization has declared *H.pylori* a carcinogen, predisposing infected persons to gastric cancer and lymphoma. Several oral antimicrobial agents have efficacy against *H. pylori*. Antibiotic treatment for *H. pylori* infection is often accompanied by side effects including development of resistance to antimicrobial agents. Considerable interest has focused recently on alternative/adjunct approaches such as biologically active compounds including antioxidants from plants and other natural sources. Previous publications have focused on the role of *H. pylori* in production of oxygen-free radicals such as superoxide anion and hydroxyl radicals, which lead to increased oxidative damage (a major cause of gastric injury), as demonstrated by enhanced lipid peroxidation and increased DNA damage in the gastric tissues. Natural antioxidants may thus serve as novel therapeutic tools in alleviating *H. pylori*-induced oxidative damage (Archana et al., 2004).

Many hundreds of worldwide plants are used in traditional medicine as treatments for different kinds of diseases including bacterial infections and gastrointestinal disorders. Among these bacteria, *H. pylori*, a Gram-negative pathogenic bacterium which specifically colonizes the human gastric mucosa, has been regarded as a primary causative agent of chronic gastritis and peptic ulcer diseases including mucosa-associated lymphoid tissue lymphoma. The most effective therapy is still unknown and prompts people to make great efforts to find better and more modern natural or synthetic anti-*H. Pylori*agents (Biglar et al., 2012; Ge & Sun., 2012).

Urease (EC 3.5.1.5, urea amidohydrolase), a nickel-dependent metalloenzyme, catalyzes the hydrolysis of urea to form ammonia and carbon dioxide (Kumar & Kayastha, 2010). While *H. pylori* is acid sensitive

and only replicates at pH of 7-8, it survives in the stomach under highly acidic conditions (5-7) urease activity in bacteria is believed to be essential for the colonization and survival of *H. pylori* at very acidic pH. Thus virulence of *H. pylori* could be controlled using chemicals that inhibit urease activity (Biglar et al., 2012). Current efforts focus on the discovery of new urease inhibitors is against *Helicobacter pylori* urease.

Citrullus colocynthis (Bitter apple) dependent to the family of *Cucurbitaceae*, which grow abundantly along the arid area of Southwest of Asia. Although, the whole fruit is often used for the treatment of the aforementioned diseases, but some particular parts of the fruit are also used for specific purposes. One of such example is the traditional application of the dried pulp and seed extract of *C.colocynthis* for the therapy of constipation and diabetes (Marzouk et al., 2011; Satyavani et al., 2011; Tannin-Spitz et al., 2007). This report is focused on *Citrullus colocynthis* extracts as natural antioxidant and urease inhibitors that can be used directly or as lead compounds in management of *H.pylori* infection.

Materials and Methods

- **Preparation of the pulp extract and root extract from Citrullus colocynthis**

The fruit and root of the plant were collected from Kerman province,Iran, during the month of June , 2012. Botanical identification was performed by Dr. A. Naqinejhad, Department of Biology, Mazandaran University, where the voucher specimen has been deposited. In our labor the different parts of *Citrullus colocynthis* (including pulp and roots) in powdered form were extracted with methanol using a Soxhlet assembly for 24h, filtered and last traces of the solvent were evaporated under reduced pressure in a rotary evaporator (Kumar et al., 2008).

- **Chemicals**

Ascorbic acid, quercitin, gallic acid, sodium phosphate, ammonium molybdate, trichloro acetic acid (TCA), sulphuric acid, sodium nitrit5%, aluminium chloride 10%, sodium hydroxide, $FeCl_3$, potassium ferricyanide $K_3[Fe(CN)_6]$, methanol and acetone were from sigma chemical company (Germany). All other chemicals and reagents were obtained from Fluka BioChemika and Merck companies.

- **Determination of total phenol and flavonoid content**

The total phenolic content of *Citrullus colocynthis* pulp (CCP) and root (CCR) extract, was determined according to the method of Singleton and a solution of gallic acid was used as standard (Singleton & Rossi., 1965). Total flavonoid contents were measured with the aluminum chloride colorimetric method (Kumar et al., 2008). The calibration curve was prepared by preparing quercitin solutions at 10-100 mg/ml in methanol.

- **Reducing Power potential**

The ability of the extracts to reduce iron (III), which is an important mechanism of phenolic antioxidant action, was assessed by the method of Yildirim (Yildirim et al., 2001). The methanolic pulp and root extracts (100-600 µg) in 1 ml of distilled water and methanol were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide $K_3[Fe(CN)_6]$. The mixture was incubated at 50°C for 30 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$. The absorbance was measured at 700 nm. High absorbance indicates high reducing power. Ascorbic acid was used as positive control (Yildirim et al., 2001).

- **Total antioxidant capacity determination**

The antioxidant activity of extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto (Prieto et al., 1999). An aliquot of 0.1 ml of sample solution was combined with 1ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. After cooling the samples in room temperature, the absorbance of each test tube was measured at 695 nm. Ascorbic acid was used as standard and all the measurements were carried out in triplicate and the results were mean value.

- **DPPH free radical scavenging activity**

The ability of extracts to scavenge DPPH radicals was determined according to the method of Blois (1958). 1 ml of a 1 mM methanolic solution of DPPH was mixed with 3 ml of extract solution in methanol (containing 50–400µg/ml of dried extract). The mixture was then vortexed vigorously and left for 30 min at room temperature in the dark (Blois., 1985). The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging related to control using the following equation:

$$\textit{DPPH scavenging activity\%} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Ascorbic acid was used as standard. All experiments were conducted in triplicate and the results were mean value.

- **Urease Extraction**

100 g of powdered soybean flour in a beaker was stirred with 500 ml of 32% acetone for 4 min at room temperature. Then the filtrate was maintained overnight at 4°C. The urease crystals were separated by centrifugation (15 min, 6000 rcf). Then the re-crystallized urease was dissolved in 3 ml of distilled water and stored in -70°C until used. Protein concentration was determined by using Lowry method with bovine serum albumin as standard (Hanif et al., 2012).

- **Urease inhibition activity assay**

The assay mixture, containing 100 µL (2 mg/mL) of soybean urease and 100 µL of the test compound with 200 µL of 100 mM phosphate buffer pH 6.8 containing 25 mM urea was pre-incubated for 30 min in water bath at 37°C. All reactions were performed in triplicate via phosphate buffer in a final volume of 0.4 mL. The urease reaction was stopped after 30 min incubation with 600 µL of 4% H_2SO_4. Assay of enzyme inhibition performed by Berthelot alkaline phenol–hypochlorite method. This method is based on the released ammonia which reacts with hypochlorite to form a monochloramine (Weatherburn., 1967). This product then reacted with phenol to form blue-colored indophenols whose absorbance was measured at 625 nm. The liberated ammonia was estimated using 500 µL of solution A (contained 5.0 g phenol and 25 mg of sodium nitroprusside) and 500 µL of solution B (contained of 2.5 g sodium hydroxide and 4.2 mL of sodium hypochlorite in 500 mL of distilled water) at 37°C for 30 min and the absorbance was measured at 625 nm against the control (Biglar et al., 2012). Percentage of inhibition was calculated using the formula: ((OD control - OD sample / OD control) × 100). The concentration of compound that inhibited the hydrolysis of substrate by 50% (IC_{50}), was determined through monitoring the inhibition effect of various concentrations of extracts in the assay. The IC_{50} values were then calculated using above mentioned formula in the previous section. Thiourea was used as the standard inhibitor of urease.

Results

- **Extraction yield and total phenolic (TPC) and total flavonoid contents (TFC)**

The yield of crude extracts, TPC and TFC are shown in table 1. As can be seen there, the higher amount of components in pulp was extracted in methanol (24.49%, w/w) while the lower amount of components was extracted in roots (8.94%, w/w). Then methanol was more effective for extraction of components from pulp than root of *C. colocynthis*. Phenolic compounds extracted from plants are very important because of their antioxidant activity due to their hydroxyl groups attached to aromatic rings. Therefore, it is important to measure TPC in natural extracts. In this report, total phenolic content (TPC) was measured by Folin-Ciocalteaue Reagent, and gallic acid was used as standard, and results are shown in table 1. As can be seen in this table, the higher amount was found in the methanolic extract of roots (0.023±0.003mg GAE/g dried extract) than the methanolic extract of pulp (0.014±0.005mg GAE/g dried extract). In comparison between pulp and root parts, the methanolic extract of roots also has a higher amount of total flavonoid content (1.22±0.003mg QE/g dried extract) than the methanolic pulp extract (1.00±0.002mg QE/g dried extract). These values were determined with the aluminium chloride colorimetric method.

Table 1. Total phenol and flavonoid contents of methanolic pulp and root extracts of *C.colocynthis.*

Plant part	Extraction yield (w/w %	Total phenol content mgg^{-1*}	Total flavonoid contant mgg^{-1}**
CCR	8.94	0.023±0.003	1.22±0.003
CCP	24.49	0.014±0.005	1.00±0.002

* mg gallic acid equivalent / gram dried extract

** mg quercitin equivalent / gram dried extract

Values are means of three determinations ± standard deviation.

Abbreviations: CCR, *Citrullus colocynthis* root extract; CCP, *Citrullus colocynthis* pulp extract

- **Reducing power of *C. colocynthis***

In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe^{+3} to Fe^{+2} by donating an electron. The amount of Fe^{+2} complex can be monitored by measuring the formation of Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose-response columns for the reducing powers of the extracts also increased with the increase of their concentrations. In a comparison between CCP and CCR extracts, the methanolic CCR showed the stronger reducing power than CCP extract. However, the reductive ability of the extracts was found to be low when compared to ascorbic acid as a standard. Nevertheless, these extracts have shown higher activities than control (Yildirim et al., 2001).

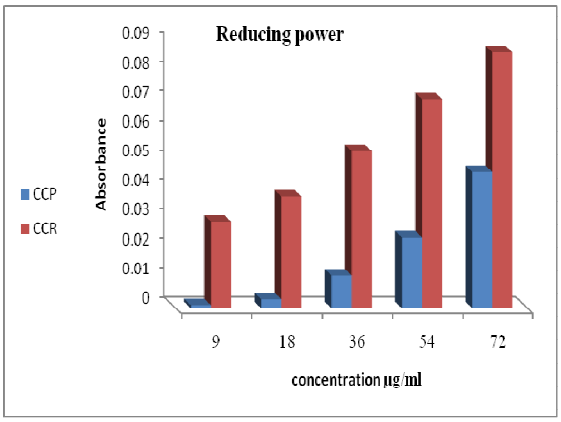

Fig.1. Reducing power of the methanolic pulp and root extracts of *Citrullus colocynthis* at different concentrations, as measured by means of spectrophotometric detection of the Fe^{3+} - Fe^{2+}.Each value represents amount±SD (n=3). For abbreviation refer to table 1.

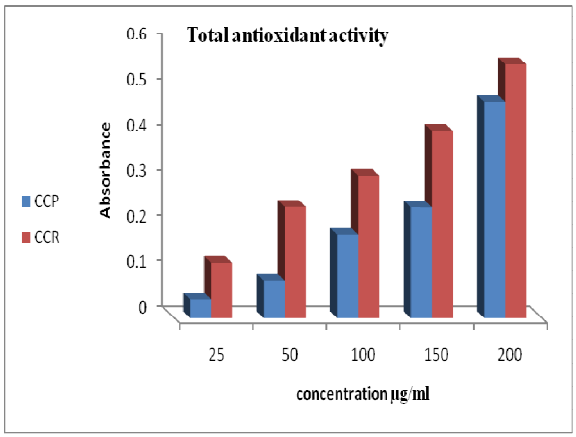

Fig.2. Total antioxidant activities of the methanolic pulp and root extracts of *Citrullus colocynthis*

- **Total antioxidant activity**

This assay is based on the reduction of Mo^{+6} to Mo^{+5} by a reductant (antioxidant) with the formation of green phosphate/Mo (V) complex, which shows a maximum absorbance at 695 nm. As given in Fig. 2, both extracts showed increasing antioxidant activity with increased concentration. In this study the methanolic CCR extract showed the stronger antioxidant activity than CCP extract. This could be due to the difference in concentrations and type of antioxidative compounds present in these extracts (Prieto et al., 1999).

- **DPPH free radical scavenging**

The DPPH method is economic, simple, rapid, and widely used to determine the antioxidant activity of phenolic compounds in natural products. In the DPPH radical scavenging assay, the extracts showed a dose-dependent moderate DPPH radical scavenging activity, and the maximum scavenging activity of 46.64% and 28.57% for CCR and CCP was found at 100 µg/ml. The ability of extracts to scavenge of DPPH radical (IC_{50}) was determined for ascorbic acid (AA), butylated hydroxytoluene (BHT), CCR and CCP, 1.72 ± 0.001 µg/ml, 8.84 ± 0.001µg/ml, 110.80 ± 0.002µg/ml, 159.07 ± 0.002 µg/ml respectively (Fig.3). However, scavenging activity of ascorbic acid and BHT, known antioxidants, used as positive controls, was relatively more pronounced than that of CCR and CCP.

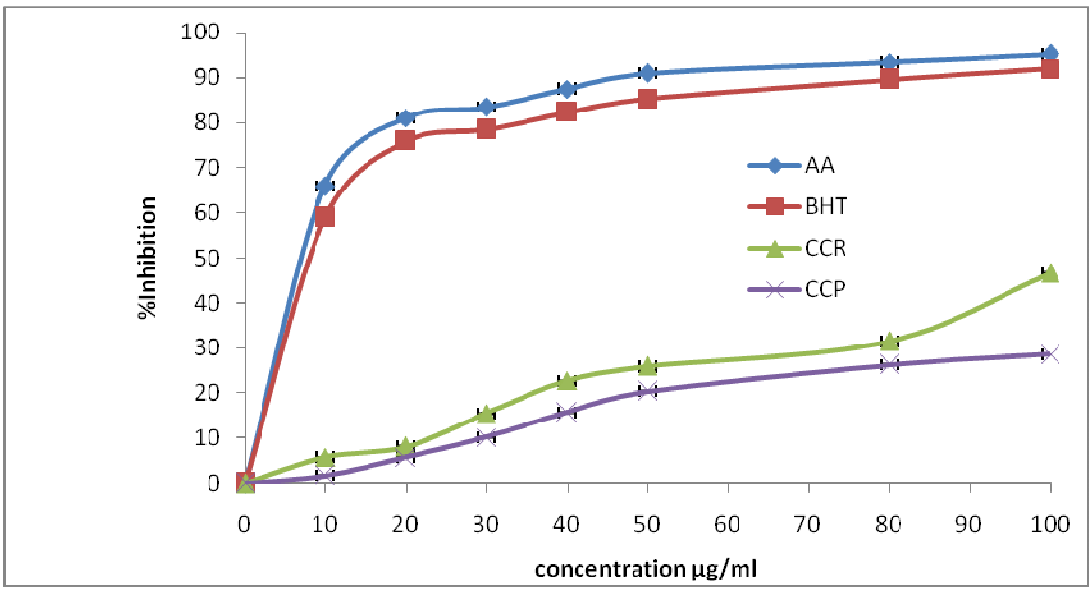

Fig.3. Scavenging effect of the *Citrullus colochnthis* extracts on DPPH radical. Each value represents a mean±SD (n=3). BHT and ascorbic acid were used as positive controls.

- **Urease inhibition activity**

It can be concluded from the previous research that the methanolic seed extract of *Citrullus colocynthis* showed antioxidant activity by free radical scavenging and quenching property and the seeds can be used as potent source of natural antioxidant and antiulcerogenic potential (Gill et al., 2011). As shown in Figure 4, concentration dependent activities against soybean urease were observed for methanolic CCP and CCR extracts and inhibitory effect increased together with increasing the concentration of each extracts in the range of (0-100µg/µl).

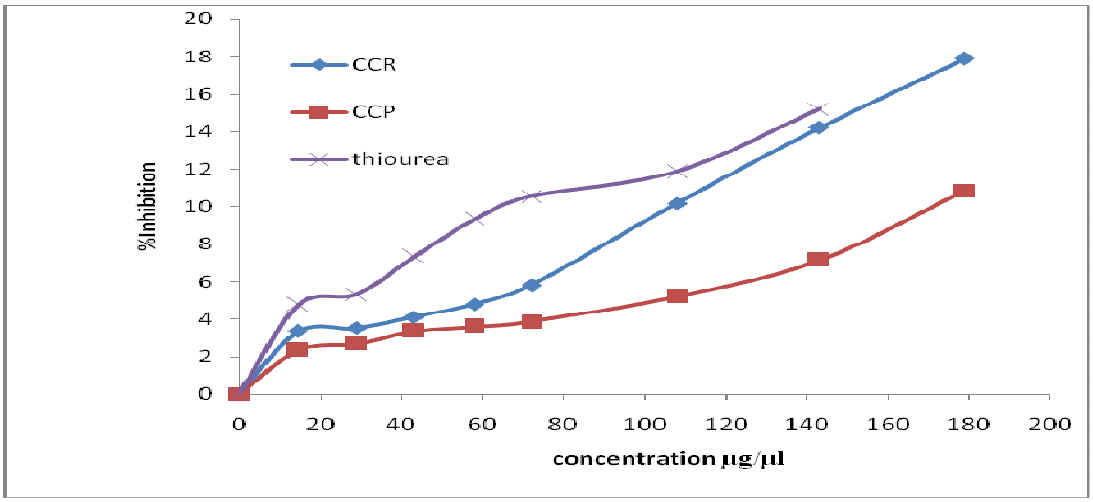

Fig.4. Inhibition profile of the methanolic pulp and root extracts of *Citrullus colocynthis* against soybean urease activity by indophenol method. Thiourea was used as positive control in urease activity assay.

As shown in table 2, the inhibitory activities of both extracts were found to be the potent inhibitors with IC_{50} = 7.31 µg/µl for CCR and IC_{50} = 13.71 µg/µl for CCP. Medicinal plants serve as a useful source of novel drugs. In developing countries, since the application of antibiotics is still under a poor management as a whole, there is a growing need for finding new medicinal plants especially anti-*H.pylori* agents that can help eradicate the invasion and presence of survived *H.pylori* strains to avoid relapse of gastric ulcer. In this regard, the literature has reported extracts of certain plants such as cashew apple, cinnamon and Chinese tea inhibit growth of *H.pylori* and some urease inhibitory activity (Biglar et al., 2012).

Table 2. The IC_{50} of urease enzyme inhibition in the presence of methanolic pulp and root extracts of *Citrullus colocynthis*.

Plant extract	IC_{50} (µg/µl)
CCP	13.71
CCR	7.31

Discussion

In previous works, it was shown that the increase of the flavonoid content associates with the increase of the free radical scavenging activity. The methanolic pulp extract of *Citrullus colocynthis* contained some flavonoids and showed moderate-high free radical scavenging activity when tested by DPPH scavenging method (Sithisarn et al., 2005). In our work, the methanolic *Citrullus colocynthis* root extract showed higher content of phenol and flavonoid components than the pulp. Antioxidant activity may be due to phenolic compounds in CCR but further work should be done on the isolation and identification of other antioxidant components of *Citrullus colocynthis*. Also, the evaluation of urease inhibitory activity of *C. colocynthis* revealed that the methanolic extract of root has considerably more activity than pulp extract and comparable with thiourea as standard inhibitor. The results of this report revealed that the *Citrullus colocynthis* could lead to introducing new candidate for further studies which, in the end, would be helpful for enhancing human health.

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