Extraction and identification of phenol compounds from Bitter Melon *Momordica charantia* fruits and their role as antioxidants

استخلاص وتشخيص بعض المركبات الفينولية من ثمار نبات الكيريلا *Momordica charantia* وفعاليتها المضادة للأكسدة

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Abstract

The antioxidant and free radical scavenging activities of Bitter Melon *Momordica charantia* exracts including phenolic compounds, ethanolic and aqueous were studied. Phenolic compounds were extracted, purified and identified by High Performance Liquid Chromatography (HPLC) method. The main phenolic constituents, which were present in the fruit extract of bitter melon, were gallic acid, protocatechuic acid, gentisic acid, catechin, chlorogenic acid and epicatechin. The results clearly indicated that phenolic compounds have an effective antioxidant activity by using Ferric Thiocyanate (FTC) method. Phenolic compounds caused 91.25% lipid peroxidation inhibition of linoleic acid emulsion. This activity was greater than ethanolic extract 82.5%, α- tocopherol 70% and aqueous extract 49.58%. Also the phenolic compounds revealed obvious activity for H_2O_2 scavenging 68.8% in comparison with α- tocopherol 45.3%, ethanolic extract 52.6% and aqueous extract 36.2%. These results confirmed the important role of phenolic compounds as antioxidants and the most antioxidant activity of bitter melon fruits belong to these compounds.

المستخلص

درست الفعالية المضادة للاكسدة والفعالية المزيلة للجذور الحرة لمستخلصات ثمار الكيريلا *Momordica charantia* (المركبات الفينولية ، الايثانولي والمائي) ، كما أستخلصت ونقيت المركبات الفينولية وشخصت باستخدام طريقة الكروماتوكرافيا السائلة عالية الأداء . شخصت المكونات الفينولية الاساسية في مستخلص ثمار الكيريلا مثل حامض الكالك ، حامض البروتوكاتجويك ، حامض الجنتسك ، الكاتجين ، حامض الكلوروجنك والايبيكاتجين . أشارت النتائج بشكل واضح الى ان المركبات الفينولية في مستخلص ثمار الكيريلا أمتلكت فعالية شديدة مضادة للأكسدة بأستخدام طريقة ثايوسيانات الحديديك . ثبطت المركبات الفينولية المستخلصة أكسدة الدهون لمستحلب حامض اللينوليك بنسبة 91.25% وهو أعلى من المستخلص الأيثانولي 82.5% والألفاتوكوفيرول 70% والمستخلص المائي 49.58% ، كذلك أظهرت المركبات الفينولية فعالية واضحة لأزالة بيروكسيد الهيدروجين وبنسبة 68% مقارنة بالألفاتوكوفيرول 45.3% والمستخلص الأيثانولي 52.6% والمستخلص المائي 36.2% . هذه النتائج اكدت الدور المهم للمركبات ا لفينولية كمواد مضادة للاكسدة وان معظم الفعالية المضادة للاكسدة في ثمار نبات الكيريلا تعود لهذه المركبات .

Key words: *Momordica charantia*, Phenolic compounds, Antioxidants.

Introduction

Bitter melon *M. charantia* is a herbal plant belongs to the family cucurbitaceae Figure (1). Also known as bitter gourd, karela [1].

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Fig (1): Fruits of bitter melon *(M. charantia*)

It has been commonly consumed as a vegetable and used as a medicinal herb in India, China, Africa and various parts of Asia. Past reports depict that it is helpful in treating wound, ulcer, eczema, jaundice, kidney stone, leprosy and scabies [2]. The main constituents of bitter melon which are responsible for medicinal effects are triterpenes, steroids, alkaloids, inorganics, lipids and phenolic compounds [3].

Phenolic compounds are categorized as secondary metabolites essential for growth and reproduction of plants. They are known as hydrophilic antioxidants, and are produced as a response for defending injured plants against pathogens, they potentially show antioxidant, antimutagen, antitumer, anti-inflammatory and anticarcinogenic properties [4]. Free radicals are known to be the major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, diabetes mellitus and cancer [5]. Recently, natural foods and food-derived antioxidants such as vitamins and phenol phytochemicals have received growing attention because they are known to function as chemoprotective agents against oxidative damage [6].

The main objectives of this study were extraction and purification of phenol compounds from Bitter melon (*M. charantia*); analyze them by HPLC (High Performance Liquid Chromatography) and evaluation the antioxidant activity and scavenging activity of the phenol extract in comparison with the crude ethanol and aqueous extracts in order to justify their therapeutic use.

Materials and methods

Plant material

Fresh fruits of *M. Charantia* were procured from markets of Arbil city, North of Iraq. Authentication and identification of the plant was carried out by Prof. Dr. Ali Al-Mosawy, Department of Biology, College of Science, University of Baghdad.

Sample preparation

Fruits of bitter melon were cleaned and cut into small pieces, and then oven dried at 50^{o}C for a day. The dried sample was then pulverized into fine powder in a grinder, and then stored at 4^{o}C until use.

Extraction and purification of phenolics [7]

A dried sample of bitter melon l0 g extracted for 30 min. by stirring at 4°C with 200 ml of cold aqueous ethanol %65 containing 0.5% Sodium metabisulphite. The homogenate was filtered through four layers of cheesecloth, and the residue was then extracted with two additional portions (100 ml each) of the same extraction solution as described above. The combined filtrate was centrifuged at 7000 rpm for 15 min. at 4°C and residue was discarded. Ethanol was removed from the supernatant by rotary evaporator under vacuum at 35°C, and the mass is measured. Pigments were eliminated by two successive extractions with petroleum ether. After addition of 20% ammonium sulphate and 2% metaphosphoric acid to the aqueous phase, the compounds were extracted three times with ethyl acetate. The extracts were combined, evaporated and then dried under vacuum at 35°C. The residue was redissolved in methanol (1:1) for analysis.

Determination of phenolic compounds

The phenolic compounds of the bitter melon were determined using High Performance Liquid Chromatography (HPLC) [8]. The absorbance was monitored at 254 nm.C-18 Chromatographic column was used. The mobile phase consisted of 100 % methanol. A sample size of 5 µl from the intact phenolics was injected for the HPLC analyses.

Preparation of extracts [6]

The aqueous bitter melon extract was prepared by extracting l00 g of bitter melon powder with 1L of boiling water for 1 hr. The extract was filtered with filter paper (Whatman No.1), the residue was then re- extracted under the same conditions twice. The filtrates were combined, evaporated to dryness by rotary evaporator and the remaining mass is measured.

Ethanol extract was prepared by soaking 100g of bitter melon powder with 1L of ethanol 95% at room temperature for 48 hrs, filtered with a filter paper (Whatman No.1). The filtrate was collected, combined, evaporated to dryness by using rotary evaporator and the remaining mass is measured. For both extracts, the dried powdered – extract was stored at 4°C until use. The percentage of yield is calculated as mg per g dried fruits.

Antioxidant activity of bitter melon extracts

Antioxidant activity of bitter melon extracts and standards was determined by FTC (Ferric Thiocyanate) method [9].

For preparation of stock solutions, 10 mg of each extract of bitter melon extracts (phenolic compounds, aqueous and ethanolic) was dissolved in 10 ml of distilled water. Then, the solution which contained 50 µg/ml of stock bitter melon solution or α- tocopherol as standard sample (50 µg /ml) in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0), was added to 2.5 ml of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). The mixture 5ml was incubated at 37°C in a flask. The peroxide level was determined at absorbance of 500nm, after the reaction with $FeCl_2$ and thiocyanate at intervals during incubation. During the linoleic acid oxidation, peroxides were formed, which oxidized Fe^{+2} to Fe^{+3}.

The latter Ions formed a complex with thiocyanate and this complex showed maximum absorbance at 500 nm. Five ml linoleic acid emulsion: contained 17.5 µg Tween-20, 15.5 µl Linoleic acid and 0.04 M potassium Phosphate buffer (pH 7.0). On the other hand, 5 ml control was composed of 2.5 ml linoliec acid emulsion and 2.5 ml 0.04 M potassium phosphate buffer (pH 7.0). This step was repeated every 5 hrs. until the control reached its maximum absorbance value. Therefore, high absorbance indicates a high linoleic acid emulsion oxidation. Solution without added extracts was used as blank samples. All data on total antioxidant activity were the average of duplicate analyses. The percentage inhibition of lipid peroxidation in linoleic acid emulsion was calculated by using the following equation:

Inhibition of lipid peroxidation(%)

$$= 100 - \left[\frac{\boldsymbol{A}sample}{\boldsymbol{A}control} \times 100\right]$$

Where: $\boldsymbol{A}\ control$ is the absorbance of the control reaction.

$\boldsymbol{A}\ sample$ is the absorbance of the presence of the sample of (phenolic, aqueous or ethanolic extract or standard compound (α- tocopherol) .

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity of bitter melon extracts (phenolics, ethanolic and aqueous) was determined according to the method of [10]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (50 µg/ ml) in distilled water were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contained the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of bitter melon extracts and standard compound (α- tocopherol) was calculated as:

H_2O_2 scavenging effect (%)

$$= \left[\frac{\boldsymbol{A}control\ -\boldsymbol{A}sample}{\boldsymbol{A}control}\right] \times 100$$

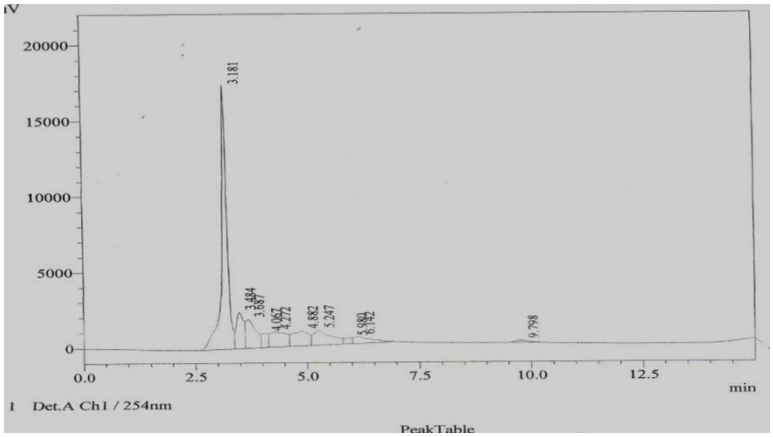
Where: $\boldsymbol{A}control$ is the absorbance of the control.

$\boldsymbol{A}sample$ is the absorbaunce of the sample of extracts and standard.

Results and Discussion

Phenolic compounds extract of bitter melon constituents were determined by using HPLC.

Phenolic constituents of the sample identified by their retention times, and the comparison of the result of HPLC of phenolic compounds extract of bitter melon Figure(2) with HPLC profiles of 14 standard phenolics Figure (3) as described in [11]. The main phenolic constituents, which were present in the fruit extract of bitter melon, were gallic acid, protocatechuic acid, gentistic acid, catechin, chlorogenic acid and epicatechin.



Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.181	154147	17413	48.604	65.988
2	3.484	27434	2363	8.650	8.955
3	3.687	30257	1867	9.540	7.076
4	4.067	9435	922	2.975	3.494
5	4.272	25327	993	7.986	3.762
6	4.882	24434	963	7.704	3.650
7	5.247	27348	956	8.623	3.621
8	5.980	4399	359	1.387	1.361
9	6.142	11852	435	3.737	1.649
10	9.798	2518	117	0.794	0.444
Total		317150	26388	100.000	100.000

Fig (2): High Performance Liquid Chromatography of bitter melon phenolic compounds extract

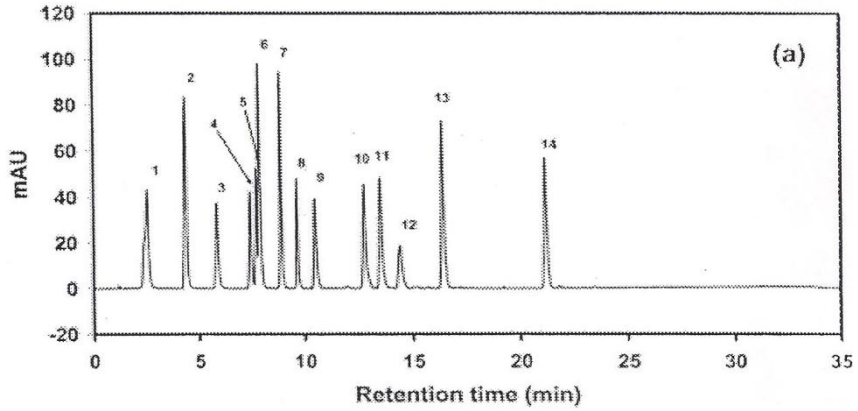


Fig (3): HPLC profiles of 14 standard phenolic acids. Peaks: 1, gallic acid; 2, Protocatechuic acid; 3, gentisic acid; 4, catechin; 5, Vanillic caid ; 6, chlorogenic acid; 7, syringic acid; 8, epicatechin; 9 p-coumaric acid; 10, t-ferulic acid; 11, sinapic acid, 12, benzoic acid; 13, o-coumaric acid; ; 14, t- cinnamic acid. Horax *et al*. (2005) [11]

Bitter melon has high contents of phenolics such as gallic acid, gentisic acid, catechin and epicatechin[12].

The antioxidant activities of bitter melon extracts and standard compound (α-tocopherol) at the concentration 50 µg/ml were showed in Figure (4).

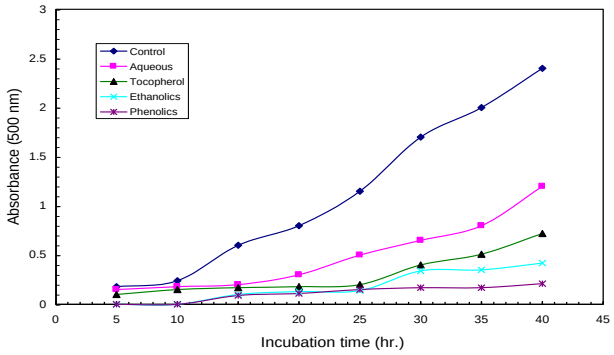


Fig (4): Antioxidant activities of bitter melon extracts (phenolics, ethanolic and aqueous) and standard compound (α- tocopherol) at the concentration 50 µg/ml.

After 40 hr. of incubation, the phenolic compounds of bitter melon caused 91.25 % lipid peroxidation inhibition of linoleic acid emulsion, this activity was greater than ethanolic extract 82.5 %, α- tocopnerol 70 % and aqueous extract 49.58%. These results clearly indicate that phenolic compounds have an effective and powerful antioxidant activity by FTC method. On the other hand it was obvious that the lowering of antioxidant activity of aqueous extract of bitter melon in comparison with the standard compound and other extracts. This may be due to the destruction of phenolic compounds by heat during extraction. Some studies showed a high correlation between antioxidant activity and total phenolics and that antioxidant effect of many natural plant extracts is related to their phenolics [11,13].

Hydrogen peroxide (H_2O_2) Scavenging activity of bitter melon extracts (phenolic compounds, ethanolic and aqueous) were summarized in Table (1). The results were compared with α- tocopnerolas standard compound.

Table (1): Scavenging activity of bitter melon extracts (phenolics, ethanolic and aqueous) and standard antioxidant compound (α- tocopherol) at concentration 50 µg, ml.

Type of compound	H_2O_2Scavenging activity (%)
α- tocopherol	45.3 %*
Phenolic compounds extract	68.8 %
Ethanolic extract	52.6 %
Aqueous extract	36.2 %

The Values are given as mean of duplicate.

Phenolic compounds at 50 µg/ml, ethanolic extract and aqueous extract exhibited 68.8 %, 52.8 % and 36.2 % scavenging activity of H_2O_2, respectively. On the other hand, α- tocopherol revealed 45.3 % Scavenging activity at the same concentration. These results showed that α- tocopherol had lower Scavenging activity in contrast with phenolics and ethanolic extract, and the phenolic compounds showed the stronger scavenging activity. The scavenging of H_2O_2 by extracts may attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water [14, 15]. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells because it may give rise to hydroxyl radicals in the cells. Addition of H_2O_2 to cells in culture can lead to transition metal ion- dependent OH radical mediated oxidative DNA- damage [16]. Thus, removing hydrogen peroxide as well as superoxide anion is very important for protection of pharmaceuticals and food products.

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