Antioxidant, Antibacterial and Cytotoxicity Activities of Flavonoid Extract From *Capsicum annum L.* Seeds

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Abstract

The present study was designed to determine the antioxidant, antibacterial and cytotoxicity activities of the flavonoid compound (**F_1**) extracted from *Capsicum annum L.* Seeds. The compound showed greater antibacterial activity against gram positive (*Staphylococcus aureus*, and *Streptococcus aureus*) bacteria than gram negative (*Klebsiella pneumonia, Pseudomonas aeruginosa and, E. coli)* using the disc diffusion method. The extract showed high activity against *Streptococcus aureus* with a zone of inhibition (40 mm) than that of *E. coli* (20 mm) at a concentration of 60 mg/ml. Antioxidant, reducing power and chelating of ferrous ion activities were also significantly higher in the flavonoid compound (**F_1**). It was found that these activities were increase as the extract concentration increases (using three concentration 15 mg/ml, 30 mg/ml & 60 mg/ml, respectively). The results showed that the flavonoid compound F_1 had no cytotoxicity against the human red blood cells in all the concentration tested (ranging between 0.5-200 ppm), by using DMSO solution as a control.

Key words: *Capsicum annum L.*, Flavonoids, Antioxidant, Antibacterial, Cytotoxicity.

ألخلاصه

صممت الدراسة الحالية لقياس فعالية المركب الفلافونيدي (**F_1**) المعزول من بذور نبات الفلفل الأخضر *Capsicum annum L.* كمضاد للأكسدة وللبكتيريا ودراسة سميته الخلوية تجاه كريات الدم الحمراء). أظهرت النتائج أن الفعالية التثبيطية تزداد بزيادة التركيز وأن البكتيريا الموجبة كانت أكثر تأثرا من البكتيريا السالبة لصبغة كرام إذ لوحظ أن

أعلى معدل لأقطار التثبيط (40 ملم) بالتركيز (60 ملغم/مل) تجاه العزلة الجرثومية *Streptococcus aureus* مقارنة مع (20ملم) تجاه *E. coli*. كما درست فعاليته كمضاد للأكسدة باستخدام ثلاث اختبارات، وقد أظهر المركب الفلافونيدي (F_1) لبذور نبات الفلفل الأخضر فعالية عاليه كمضاد لأكسدة حامض اللينولينك ، قابلية اختزال قويه وقدرة عالية للارتباط مع ايون الحديدوز في النظام النموذجي (تزداد مع زيادة تركيز الخلاصة). كذلك أوضحت نتائج الدراسة السمية عدم وجود تكتل للمركب داخل كريات الدم الحمراء حتى عند استخدام التركيز العالي (200 ppm) وباستخدام DMSO كمحلول سيطرة.

الكلمات المفتاحية : الفلفل الأخضر، الفلافونيدات، مضادات الأكسدة، مضادات البكتيريا، السمية الخلوية.

Introduction

In the last few years, there has been a growing interest in providing natural antioxidants. The protective effects of fruits and vegetables against coronary heart disease, stroke, and cancer have been attributed to the presence of flavonoids and other phytochemicals [1]. All aerobic organisms, including human beings, have antioxidant defenses that protect against oxidative damages and repair enzymes to remove or repair damaged molecules. However, this natural antioxidant mechanism could be inefficient, and hence dietary intake of antioxidant compounds is important. Recent reports indicated that there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human diseases [2]. On the other hand, natural products perform various functions, and many of them have interesting and useful biological activities [3]. The increase use or overuse of antibiotics in the treatment of bacterial infections is bringing on an increasing in pathogenic organisms that are resistant to available antibiotics. Consequently, it is necessary to increase administrated doses, combine antibiotics or provide new antibiotics withlesser tendency for pathogenic organisms to develop resistance to diseases [4]. The objective of this study was to isolate flavonoid compounds from *Capsicum annum L.* seeds and to investigate their possible antioxidant, antibacterial and cytotoxicity activities *in vitro* as a potential source of natural functional substances for using it as dietary medicinal plant.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), and solvents were from E. Merck (Darmstadt, Germany). All reagents were prepared in

deionized distilled water (DDH2O) to eliminate the contamination of metal ions.

Plant Material

Capsicum annum L. Seeds were supplied locally. The plant was botanically authenticated and voucher specimens were deposited in the Herbarium of Basrah (Iraq, Basrah, College of Science, University of Basrah). The seeds were ground by blender (Retell coffee grinder type 24) and kept in polyethylene bags at room temperature until used.

Preparation of the extracts

Aqueous extract of *Capsicum annum L.*

A suspension of 50 gm of the defatted dried powder of plant seeds in 200 mL of DDH2O was stirred magnetically for 24 hours at room temperature. The residue was removed by filtration and the filtrate was concentrated under vacum in a rotary evaporator to yield 4.32g.

Flavonoids extract of *Capsicum annum L*.

Twenty five g of dried powder leaves were extracted with 250 mL of 80% methanol by stirring at 25 °C, for 24 hours. The 80% methanol extract was filtrated, to the filtrate 25 mL of 1% lead acetate was added. The mixture was filtrated by Buchner funnel, and the precipitate treated with 25 mL acetone and 30 mL concentrated HCl. The mixture was then filtered and the filtered was evaporated to yield 0.65 g, which was dissolved in 25 mL DDH_2O, and extracted by ethyl acetate (3×50mL). The combined ethyl acetate fractions were dried by freeze drier to yield 0.23g [5].

Isolation of the components of flavonoids extract

The component **F_1** (Spot with higher R_f = 0.53) of flavonoids extract was separated and purified by column chromatography technique. A glass column size (3×60 cm) was plug down to the bottom with small glass wool, then packed with HCl-washed silica-gel (mesh 230-400 μm). The slurry was prepared by dissolving 125 g of silica-gel in 200 ml of Ethyl acetate **:** S-Butanol (1**:**1) as eluent. The solid residue was then loaded to the top of the column and fractions of 5mL/min were collected and monitored by TLC. Fractions with the similar R_f were collected and dried at room temperature [6].

Preliminary phytochemicals analysis

A Preliminary phytochemicals study (colour reactions) on all extracts was performed using standard procedures in order to determine the presence of alkaloids (Dragendroff test), carbohydrates (Molisch test), glycosides (Benedict test), saponines (Stable foam test), steroids (Liebermann-Burchard test), flavonoids (Shinoda test) and terpenoids (Salkowsky test) [7 ; 8].

Thin layer chromatography (TLC)

To detect the number of spots (chemical components) of flavonoids which are present in flavonoids extract and the flavonoid compound **F_1** of *Capsicum annum L.*, thin layer plates (2×10 cm) coated by silica gel and activated in oven at 100 °C for 1 hours were used for this purpose. Ethyl acetate **:** S-Butanol was used with ratio (1**:**1) as eluent for 15 minutes and the plates were dried using hair drier and examined under long wavelength 366nm ultraviolet. The plates were also saturated with ammonium vapor. The plates were also stained with $FeCl_3$-$K_3Fe(CN)_6$ (1:1) [9]. The plates were also stained with Antimony chloride (10% in $CHCl_3$), Folin-Denis, Benedict reagent and lead acetate (basic, 25%) [5].

Antibacterial activity test

Antibacterial activity was determined by using Mueller Hinton agar [10]. Petri plates were prepared by pouring 10 ml of Mueller Hinton agar and allowed to solidify. Plates were dried and 100 µl of inoculum's suspension (approximately 10^6 cfu/ml of bacteria) was poured and uniformly. Whatman filter paper discs(5mm) were impregnated with 2 ml of flavonoid extract at various concentration (125, 250, and 500 mg/ml) and used to fill hole bored by cork borer in the inoculated agar. All the plates were incubated at 37 °C for 24 hours to obtain maximum growth in the culture media. For each concentration, the diameter of inhibition zone of growth minus the diameter of the disc was measured to estimate the degree of antibacterial activity. The control assay was performed using ethyl acetate (0.5 ml).

Antioxidant activity test

The β-carotene bleaching method

Antioxidant activity of the flavonoid compound **F_1** of *Capsicum annum L.* seeds was determined according to the β-carotene bleaching method developed by Karadeniz [11]. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform, 1mL of this solution was then pipetted into a round-bottom rotary flask containing 20 mg of linoleic acid and 0.2 g of Tween 20. After removing the chloroform under vacuum for 4 minute using a rotary evaporator at 30 °C, 50mL of aerated distilled water was added to the flask with manual shaking. Aliquots (5 mL) of this prepared emulsion were transferred into tubes containing 0.2mL of extract (15, 30 and 60 mg/L) or butylated hydroxyl toluene (BHT, 50 mg/L) which was used for comparative purposes. The control consisted of 0.2 mL of 80% methanol instead of the extract. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. The samples were then subjected to thermal

auto-oxidation at 50 °C in a water bath. Subsequent absorbance readings were recorded at 15 min intervals until the color of the β-carotene in the control sample had disappeared (105 min). The extent of inhibition of the absorbance is related to the concentration of antioxidant compounds. The degradation rate of extracts was calculated according to zero order reaction kinetics. Antioxidant activity (AA) was calculated as percent of inhibition relative to the control using the following equation**:**

$$\mathbf{AA} = [1 - (T_1 - T_2) / (T_3 - T_4)] \times 100$$

Where:

T_1 = Absorbance of sample at zero time, T_2 = Absorbance of sample after incubation (105min) at 50 °C, T_3 = Absorbance of control at zero time, T_4 = Absorbance of control after incubation(105min) at 50 °C.

Reducing Power Method

The reductive capability of the flavonoid compound $\mathbf{F_1}$ was quantified by the method of[12]. The extract (15, 30 and 60 mg/mL) or butylated hydroxyl toluene (BHT) was mixed with an equal volume of 0.2M phosphate buffer, pH 6.6, and 1% potassium ferricyanide, then incubated at 50° C for 20 min. An equal volume of 1% trichloroacetic acid was added to the mixture and centrifuged at 3000 Xg for 10 min at room temperature. The upper layer of the solution was mixed with ddH2O and 0.1% FeCl3 with a ratio of 1**:**1**:**2, and the absorbance at 700 nm was measured.

Cytotoxicity test

The cytotoxicity activity of the extract was determined against human red blood cells using a suspension of 1ml of the blood dissolved in 20 ml of normal saline solution. Different concentrations of the extract were prepared separately dissolved in DMSO solution, then 100 µl of each concentration was added to 2ml of blood. The turbidity of the mixture was examined after 10, 30 and 60 minutes before the blood cells were haemolysate completely[13].

Statistical analysis

The results are expressed as mean values ± SD and tested with analysis of variance followed by Student's *t*-test. P-values < 0.05, < 0.01 were considered to be statistically significant.

Results and Discussion

Qualitative analysis for all extracts of *Capsicum annum L.*

Table (3-1) indicate the preliminary phytochemicals analysis for aqueous extract, flavonoid extract and flavonoid compound $\mathbf{F_1}$ of *Capsicum annum L* seeds. The results showed the presence of alkaloids, glycosides, flavonoids and saponins in the aqueous extract. The same table indicated that the flavonoid extract contained only flavonoid compounds. The most phytochemical classified as

secondary metabolites were produced mainly by the shoot part of the plant, often their functional in the plant is unknown but some phytochemicals are known to have structural, functional and general defense against plant pathogens [14]. Plant extract is the best source of phytochemical, which used as medical treatment but their uses as well as other alternative form of phyto-treatment [15]. The results presented in Figure (3-1) and Table(3-2) showed that the flavonoid extract of *Capsicum annum L.* seeds contains two components. These components are relate to the flavonoid family because they give a positive test with Lead acetate (basic, 25%), Folin-Denis, Benedict reagent, $FeCl_3$-$K_3Fe(CN)_6$ (1:1) and Antimony chloride(10% in $CHCl_3$) reagents. The results revealed that the isolated flavonoid compound **F_1** by column chromatography technique, is one compound belong to the flavonoid family.

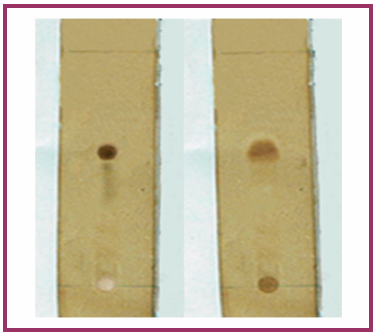

Figure (3-1): Thin layer chromatography for flavonoid extract and flavonoid compound F_1 of *Capsicum annum L.* seeds

Table (3-1): Qualitative analysis for all extracts of *Capsicum annum L.* seeds

Chemical constituent	Remarks		
	Aqueous extract	Flavonoid extract	Flavonoid compound **A**
Alkaloids	+	-	-
Carbohydrates	+		
Glycosides	+	-	-
Steroids	-	-	-
Flavonoids	+	+	+
Tannins	-	-	-
Saponines	+	-	-
Terpenoids	-	-	-

+ Present, - Absent of the chemical constituent

Table (3-2): Thin layer chromatography for all extracts of *Capsicum annum L.* seeds

UV 366 nm		Lead acetate (basic, 25%)		Antimony chloride (10% in $CHCl_3$)		Folin-Denis reagent		Benedict reagent		$FeCl_3$-$K_3Fe(CN)_6$ (1:1)	
FE	**FCF_1**	**FE**	**FCF_1**	**FE**	**FCF_1**	**FE**	**FCF_1**	**FE**	**FCF_1**	**FE**	**FCF_1**
0.53 0.01	**0.53**	**0.53 0.01**	**0.53**	**0.53 0.01**	**0.53**	**0.53 0.01**	**0.53**	**0.53 0.01**	**0.53**	**0.53 0.01**	**0.53**
Conjugated test		Flavonoid test		Flavonoid test		Flavonoid test		Flavonoid test		Flavonoid test	

FE: flavonoid Extract and **FC F_1:** flavonoid compound **F_1**

Antibacterial Activity for flavonoid compound F_1

The antibacterial activity of the flavonoid compound **F_1** of the *Capsicum annum L.* seeds was determined against 5 bacterial stains which are listed in Table (3-3). The antibacterial activity was observed to be in dose-dependent manner i.e. 60 mg/ml showed higher activity than 30 and 15 mg/ml against all tested bacteria. The results showed that the increase in the concentration of extract increased the zone of inhibition against all tested bacteria. In the present study gram positive bacteria was found to be more susceptible to the plant extract than gram negative bacteria. Our findings are in concordance with the previous reports that plant extract are more active against gram positive bacteria than gram negative one[16], which is due to the differences in their cell wall structure [10]. From the results, flavonoid compound **F_1** did not show any activity at a concentration of 15 mg/ml against *E. coli* as compared to other tested microorganisms. Interestingly, the extract showed high activity against *Streptococcus aureus* with a zone of inhibition (40 mm) than that of *E. coli* (20 mm) at a concentration of 60 mg/ml**.** From this investigation, the results obtained confirmed the therapeutic potency of *Capsicum annum L.* seeds used in traditional medicine. Moreover, these results establish a good basis for selection of the plant for further phytochemical and pharmacological investigation. The results of the present study support the folklore usage of the studied plant and recommends that the plant extract (flavonoid compound **F_1**) possess certain constituents with antibacterial properties that can be utilized as antibacterial agents in new drugs for the therapy of infectious disease caused by pathogens.

Table (3-3): Antibacterial activity of flavonoid compound F_1

Bacteria	Serial number	Diameter of inhibition zone (mm)		
		15 mg/mL	30 mg/mL	60 mg/mL
Pseudomonas aeroginosa	2	15	20	29
Klebsiella pneumonia	4	20	25	30
Staphylococcus aurens	6	20	35	40
E. coli	8	0	15	20
Streptococcus aureus	9	19	27	36

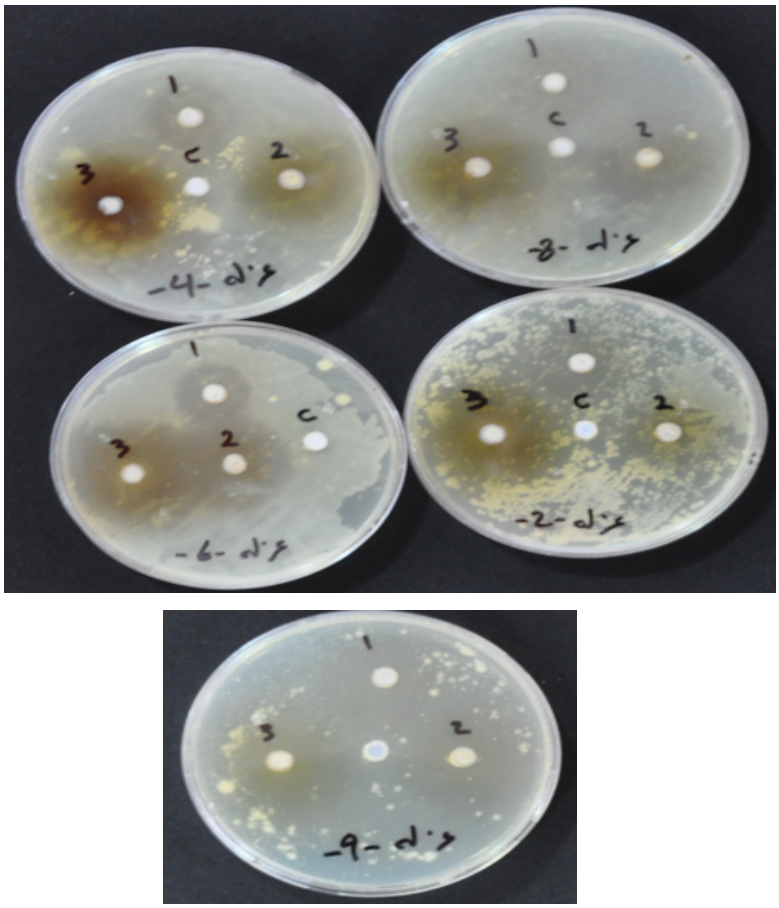

Figure (3-2): A typical agar plate showing the inhibition zones exhibited by flavonoid compound F_1 against all tested bacteria with concentration of (15, 30 & 60 mg/mL).

Antioxidant activity for flavonoid compound F_1

The results of this study for antioxidant and reducing power tests of flavonoid compound **F_1** are presented in Figures 3-2 and 3-3, respectively.

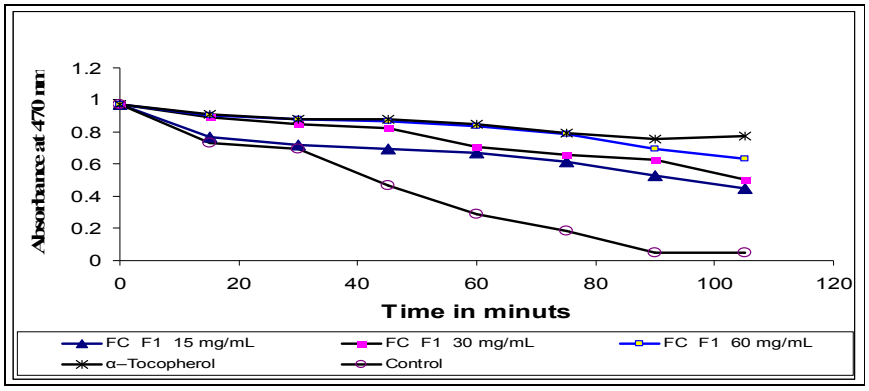

Figure (3-2): Antioxidant activity of the flavonoid compound F_1 with compared to α-Tocopherol and control .

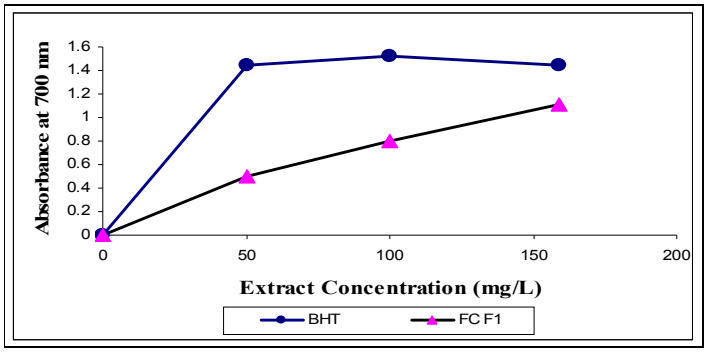

Figure (3-3): Reducing power of the flavonoid compound F_1 with compared to BHT.

The results revealed that the inhibition of peroxidation was progressively increased by raising the extract concentration of flavonoid compound **F_1** and reached a plateau (about 81.4% inhibition) when the concentration exceeded 60mg/mL ($P < 0.01$). The material extracts had overall good antioxidant activity. Flavonoid are phenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity [17]. The phenolic compounds might contribute directly to antioxidative action [18]. The antioxidative activities observed could be ascribed both to the different mechanisms exerted by different phenolic compounds and to the synergistic effects of different compounds. The antioxidant assay used in this study measures the oxidation products

at the early final stages of oxidation [19]. The antioxidants have different functional properties, such as reactive oxygen species scavenging, inhibition of generation of free radicals and chain-breaking activity and metal chelating [20]. A direct correlation between antioxidant capacity and reducing power of the extract has been reported [21]. The reducing power for flavonoid compound **F_1** of *Capsicum anumm L.* seeds was very potent and the reducing power of the extract was increased with quantity of sample. The plant extract could reduce the most Fe^{+3} ions, which had lesser reductive activity than the standard of BHT. The reducing properties are generally associated with the presence of reductions, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [22]. Therefore, antioxidants with free radical scavenging activities might have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals[23].

Cytotoxicity activity of flavonoid compound F_1

The results in Table (3-4) and Figure (3-4) showerd that the flavonoid compound F_1 had no cytotoxicity against the human red blood cells in all the concentration tested (ranging between 0.5-200 ppm), using DMSO solution as a control. The compound showed no aggregation inside the blood cells. Therefore, the effect of flavonoid compound F_1 in their cytotoxicity was differ according to each types of antibiotics specially the nucleoside antibiotics such as Tubercidine.

Table (3-4): The cytotoxicity of flavonoid compound F_1

Compound	Concentration (ppm)	Toxicity against RBC
DMSO	-	NT
Flavonoid compound **F_1**	0.5	NT
	10	NT
	50	NT
	100	NT
	200	NT

NT: non toxic, DMSO: dimethyl sulfoxide.

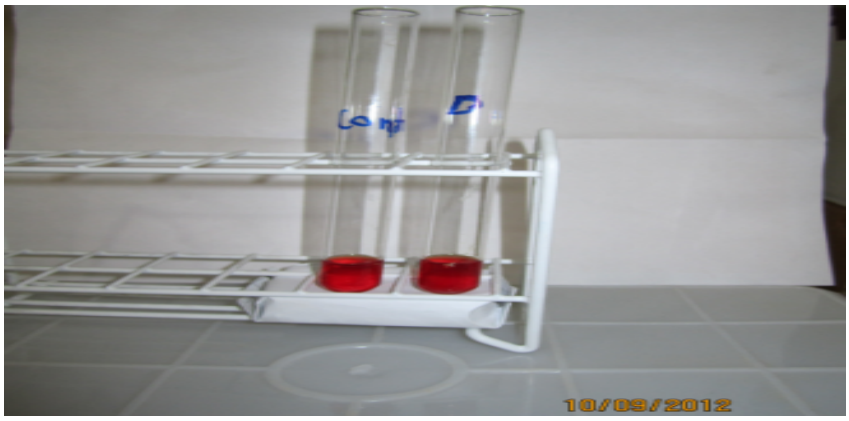

Figure (3-4): The cytotoxicity of flavonoid compound F_1

Conclusion

Based on the results of the present study, it can be concluded that the flavonoid compound **F_1** in the *Capsicum anumm L.* seeds possesses strong antibacterial and antioxidant potentials. Further studies are necessary to identify the chemical structure of this compound (**F_1**) and to elucidate the mechanisms behind its effects**.**

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