Research Article

Evaluation of an *In vitro* Antibiotic and Antioxidant Activities of Cynarin and Chlorogenic acid Extracted from Artichoke Plant Cultivated in Iraq

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ABSTRACT

The present study examined the antibiotic and antioxidant effect of cynarin and chlorogenic acid which was extracted from the leaves of Cynara scolymus (artichoke) cultivated in Iraq. TLC, FTIR and the melting point were employed for the identification of cynarin and chlorogenic acid. Staphylococcus Aureus, Bacillus Cereus, E. coli, Pseudomonas Aeruginosa and Candida albicans were used with disc diffusion method for an *in vitro* antibiotic study. The cynarin and chlorogenic acid showed various antimicrobial activity against the microorganisms under study. The *in vitro* antioxidant action was evaluated using sodium nitrite-induced methemoglobin formation method, where the extracted compounds exhibited a noticeable antioxidant effect.

Keywords: cynarin, chlorogenic acid, antibiotic, antioxidant.

INTRODUCTION

The artichoke (Cynara scolymus L.) initially grows in the Mediterranean region, and then it was cultured around the world. The flower and leaves are used worldwide for nutritional and medicinal purposes. The artichoke is popular, owing to its pleasant taste which is attributed to the phytochemical content that found in the green parts of the plants[1,2]. The artichoke extract has anti-hyperlipidemic effect[3], digestive effect, mobilization of energy reserves, induction of choleresis, hepatoprotective effect[4], stimulant effect for blood circulation, vascular protective[5]. Also, it has antiviral, anti-mutagenic, antimicrobial, gall stone treatment, blood glucose regulatory, anti-inflammatory, and quorum quenching activities and may be used as a food preservative[6,7]. The medicinal activity of the leaves is attributed to the presence of polyphenolic compounds, mainly those having mono and di-caffeoylquinic acids like cynarin (hydroxycinnamic acid derivative, which is an ester of quinic acid and two molecules of caffeic acid) and chlorogenic acid (ester of one molecule of caffeic acid with quinic acid). Many studies says that there is a link between the salubrious and the antioxidant effect of Artichoke extract, assuming that these effects may be related to the polyphenolic content, especially those containing mono and di-caffeoylquinic acids[8].

Although, it is not clearly understood how the antioxidants lead to bacteriostatic or bactericidal action, such properties are agreeable with the well-established double mechanism of action of polyphenolic compounds as an oxidants and antioxidants[9,10]. The aim of the present research is to evaluate the *in vitro* antibiotic and antioxidant properties of the artichoke-isolated cynarin and chlorogenic acid.

MATERIALS AND METHODS

The sabouraud dextrose agar and nutrient agar were from HiMedia Laboratories Pvt. Ltd (India), while EDTA tube from Medisave UK Ltd. All the chemicals used in this study were of high grade quality. The diethyl ether, ethyl acetate, silica gel GF254, hydrochloric acid, phosphate buffer saline and sodium nitrite were supplied by Sigma Aldrich Ltd. The formic acid and glacial acetic acid were provided by Scharlab S.L. The anhydrous methanol and dimethyl sulfoxide (DMSO) were supplied by Romil Ltd. The cynarin and chlorogenic acid standards were provided from Chengdu Biopurify Phytochemicals Ltd. The D.W. was from the laboratories of Basra college of pharmacy.

The instruments used in the present study are:

- Commercial blender, Soxhlet apparatus, Schoot Co., Germany. Rotary evaporator, (Heidolph), Heizbad WB eco Co., Germany, Oven 0-250 °C, Heraeus Co., Germany., UV transilluminator, Cleaver Co. Ltd, UK., FTIR spectrophotometer, IR Affinity-1, SHIMADSU Co., Japan, Melting point detector, BIBBY Co. Ltd, UK, Denver digital sensitive balance, TP-214, Germany., Centrifuge (Table Top), model PLC-05, Taiwan., UV-1100 Spectrophotometer, EMCLAB GmbH Co., Germany.

Extraction

The leaves of Cynara scolymus were collected from the plant which was cultivated in Iraq (Bagdad). The collected leaves were dried in shade for 15 days and then it was powdered and weighted. Two hundreds grams of these leaves were extracted with 1000 mL methanol (60%) in soxhlet apparatus for 10 hours. The solvent was evaporated to 200 mL by rotary evaporator, and then 200 mL of water were added to it. The aqueous phase was acidified to have (pH = 5) by adding diluted HCL, and then this phase was extracted three times with 200 mL diethyl ether. The ether portions were combined and evaporated to a concentrated solution[11].

Thin Layer Chromatography (TLC)

The concentrated solution obtained by extraction method was applied in a row of spots using capillary tubes on a glass plate (20cm x 20cm) which made by spreading a slurry of 30 grams silica gel GF254 with 60 mL distilled water using "TLC Coater". The plates were activated at 110 ^{o}C for one hour before use. The mobile phase used is (Ethyl acetate: Formic acid: Glacial acetic acid: Distilled water) in a ratio of (60: 4: 4: 5). Many separated bands were observed, and the spots were seen clearly under UV and visible light, these were compared with two spots of the standard cynarin and chlorogenic acid. The two silica gel zones were scrapped out, and each of which was separately transferred to a beaker and eluted with absolute methanol and filtered. The two filtrates were evaporated using rotary evaporator till dryness under vacuum. Two different precipitates, a yellowish-brown and white, were obtained.

Fourier Transform Infrared (FTIR) Spectroscopy

One mg of each of the precipitates was weighed properly, mixed thoroughly with sufficient quantity of KBr. The mixture is grinded, and then pressed to form KBr disc which then was subjected to FTIR spectroscopy.

Melting Point

The check for the melting points of the two isolated powders was conducted in order to assure their identities.

Antimicrobial Study

The microorganisms used in the antimicrobial study of the cynarin and chorogenic acid were Staphylococcus Aureus, Bacillus Cereus, (Gm +ve), Escherichia coli, Pseudomonas Aeruginosa (Gm –ve) and Candida albicans (Fungi). All media prepared as described by their company manufacturer instruction.

Sabouraud dextrose agar was used to breed C. albicans[12] and the nutrient agar media was used to rear bacteria[13]. In this study, the stock culture of bacteria was put in a nutrient agar slant and kept in frigerator at 10 ^{o}C till use. Then, they were incubated using nutrient agar at a temperature of 37 $^{\circ}$C for 24 hours. The resultant pure colonies were moved into a nutrient slant. Asymptotically, fungal stock culture was placed onto sabouraud dextrose agar slant and preserved at 10 ^{o}C in a refrigerator. Then after, the cultures were plated onto subouraud's agar and incubated for 72 hours at 37 ^{o}C. The pure fungal colonies were transferred into a subouraud's agar slant and stored at room temperature in a dark cupboard. The stock solutions of the extracted substances were prepared by dissolving 200 mg of each the yellowish-brown and the white precipitates in one mL of DMSO solvent to form the concentration of 200 mg/mL. Then after, other four concentrations (100, 50, 25 and 12.5 mg/mL) of each sample were prepared[14]. A pure culture of bacteria was spread on the surface of Mueller-Hinton agar. Small filter paper discs were sterilized by UV, impregnated with specified concentration of the samples (the precipitates) and gently pressed on to the surface of the agar. The plates were incubated overnight. After incubation, the inhibitory action was recognized by the presence of growth-inhibition zones, which can be seen on plates and it was easy to measure them.

Antioxidant

Antioxidant study was performed by using sodium nitrite-induced methemoglobin formation. Blood sample (20 mL) was taken from a healthy volunteer in a tube containing EDTA as anticoagulant. The sample was centrifuged for 10 minutes by a rate of 5000 rpm. The plasma then decanted and the sediment erythrocytes were washed with phosphate buffer saline three times.

The erythrocytes then diluted with 20 volumes of D.W., where they undergone hemolysis by the effect of osmotic pressure.

Then, centrifugation was conducted to get rid of puffy coat and cell wall of RBC. From this liquid of hemolyzed erythrocytes, three samples (2 mL of each) were taken and to which we added 0.5 mL of 0.1M solution of sodium nitrite.

The first sample is treated with 1 mL of DMSO solvent and served as control.

The second and the third were received 0.1 mg from the yellowish-brown and white precipitates respectively.

The formation of methemoglobin was monitored by UV-Visible spectrophotometer at wave length 631 nm[15].

RESULTS AND DISCUSSION

Thin Layer Chromatography

The R_F values for the isolated yellowish-brown and white precipitates compared to that of the standard cynarin and chlorogenic acid are presented in Table 1.

The obtained data state that the R_F values of the standards are close to that of the isolated precipitates. Accordingly, the yellowish-brown precipitate may be considered as cynarin and the white precipitate as chlorogenic acid.

FTIR Spectroscopy

The FTIR of the isolated chlorogenic acid and cynarin are stated in Figures (1) and (2) respectively. In the FTIR spectra of chorogenic acid, 3471.87 cm^{-1} band is attributed to stretching of the free (O-H) group.

The band at 3336.85 cm^{-1} can be corresponded to the H-bonding that accompany the dimeric form of chlorogenic acid[16].

The aromatic (C-H) stretching appears at 3059.1 cm^{-1}, whereas the aliphatic (C-H) stretching appears at 2951.09 cm^{-1}.

The 1689.64 cm^{-1} band belongs to the C=O stretching, while the 1639.49 cm^{-1} band is probably attributed to C=C

stretching. The 1188.15 cm^{-1} band may correspond to the stretching of the C-O group.

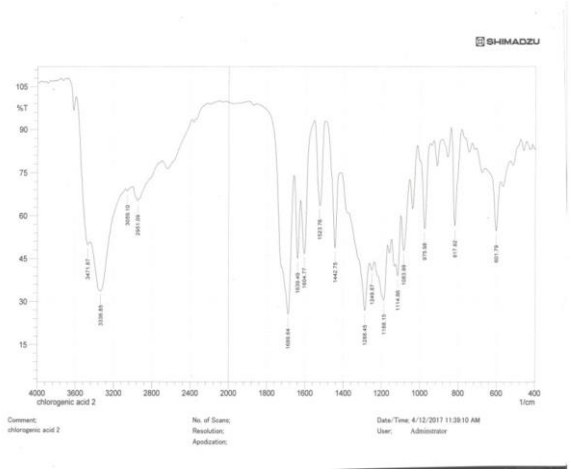

Figure 1: The FTIR of Chlorogenic Acid

In respect with the FTIR spectra of cynarin, the H-bonding of the (O-H) groups appear at 3360 cm^{-1}, while the band at 2927.94 cm^{-1} is related to the stretching of the aliphatic (C-H). The 1652.56 cm^{-1} band may correspond to C=O stretching and the band at 1026.13 cm^{-1} is related to C-O stretching.

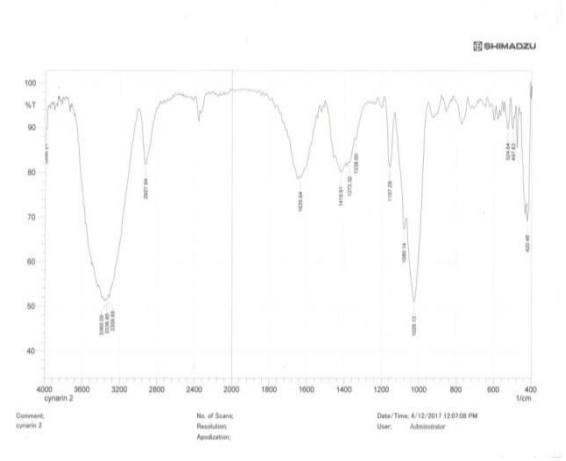

Figure 2: The FTIR of Cynarin

Melting Point

The results of the melting point determination stated that the value of the melting point for cynarin is 226-228 ^{o}C and for chorogenic acid is 207-210 ^{o}C. These values come in agreement with the references for both cynarin and chorogenic acid[17], and that give evidence in confirming the identification of these compounds.

Determination of Antimicrobial Suseptability

Gram-positive bacteria (S. aureus and Bacillus Cereus), gram-negative bacteria (E. coli) and the fungus (C. albicans) were used. Kirby Bauer disc diffusion test was employed to determine the antimicrobial susceptibility of cynarin and chlorogenic acid that isolated from the artichoke leaves.

The five concentrations (200, 100, 50, 25 and 12.5 mg/mL) of each sample were subjected to agar disc diffusion test.

Results were measured by the diameter of the inhibition zone around the disc (Table 2).

As it's clear from (Table 2), the extent of antibiotic activity (if present) of cynarin and chlorogenic is directly proportional to the concentration, so it increased with the increased concentration.

The cynarin showed no antibiotic activity in concentrations (12.5 and 25 mg/mL) for the entire tested microorganism except for P. Aeruginosa. Cynarin has an antifungal activity, but it absolutely doesn't have an activity against S. Aureus.

As it's concerned with antibacterial activity, the 12.5 mg/mL concentration of chlorgenic acid is not effective against B. Cereus and P. Aeroginosa, while the other concentrations have activity against all examined bacteria.

The chorogenic acid has no antifungal activity against C. albicans with any of the experimented concentrations. Overall, the cynarin showed some antifungal activity but no effect against S. aureus.

In contrast, chlorogenic acid showed activity against S. aureus but no antifungal effect.

Both of them show antimicrobial effects against E. coli.

It is suggested that chlorogenic acid and its related compound cynarin, have both bacteriostatic and bactericidal effects[18].

A research concerning the mechanism of action of the chlorogenic acid and its related compounds against the pathogenic microorganisms revealed that chlorogenic acid increases the cellular membranes permeability of the microorganisms, leading to loss of the membrane function.

The loss of cytoplasmic contents (like nucleotides and protein) was proved by electron micrographs.

The obtained results supported the hypothesis saying that chlorogenic acid (and the related compounds) attach to the outer cellular membrane, destroying the membrane, diminishing the intracellular potential, with loss the cytoplasmic macromolecules, which result in cell death[19].

Table 1: The R.F values of the standard and the isolated cynarin and chlorogenic acid from the artichoke.

Compound	R_F of the standard	R_F of the isolated precipitate	
Cynarin	0.83	yellowish-brown	0.81
Chlorogenic acid	0.092	white	0.09

Table 2: Inhibition Activity of Cynarin and Chlorogenic Acid for Growth of Micro-Organisms

Sample	Conc. Mg/mL	Inhibition zone diameter (mm)				
		S. aureus	*B. Cereus*	*E. coli*	*P. Aeroginosa*	*C. albicans*
Cynarin	200	-	4	9	5	6
	100	-	3	7	5	5
	50	-	-	7	4	3
	25	-	-	-	4	-
	12.5	-	-	-	2	-
Chlorogenic Acid	200	12	9	14	7	-
	100	9	8	10	6	-
	50	7	5	7	5	-
	25	6	2	6	3	-
	12.5	4	-	5	-	-
Cefuroxime	10mg/mL	14	11	12	9	-
Ketoconazole	25 mg/mL		-	-	-	18

Antioxidant Activity

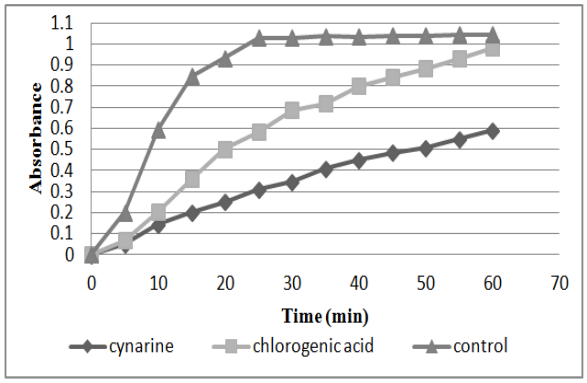

Figure 3: Antioxidant activity of Cynarin and Chlorogenic Acid versus Control

Both cynarine and chlorogenic acid showed a good antioxidant effect as compared to the control, but cynarine exerted more effect. The $t_{1/2}$ required for the control group to reach its highest value was 9 minutes, while for chlorogenic acid was about 22 minutes. In contrast, the $t_{1/2}$ for cynarine was about 60 minutes as shown in Figure 3.

That result could be explained by presence of two molecules of caffeic acid moiety, which has two hydroxyl groups in each, attached to quinine acid moiety, that can leads to stabilization of the positive charge by resonance delocalization, while in chlorogenic acid there's only one molecule of caffeic acid Figure 4.

CONCLUSION

Chlorogenic acid and cynarin can be smoothly isolated from artichoke, and they are futuristically promising biologically-active compounds. Extensive work needed to determine the suspected microorganisms which are susceptible for these agents, also to determine the optimum dose for antioxidant effect.

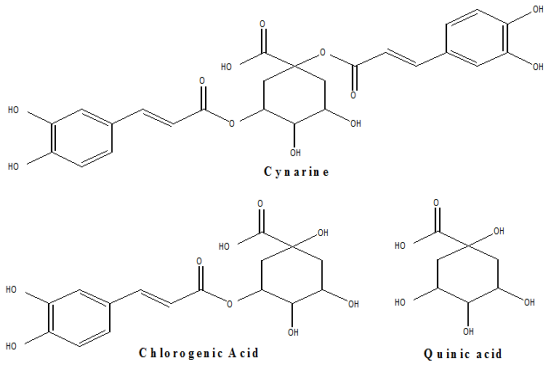

Figure 4: Chemical structures of Cynarin and Chlorogenic Acid

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