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Screening For α-Amylase Inhibitory Activity Of *Crataegus* And *Rubus* Genera In Syria, Isolation And Identification Of The Active Principle

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Abstract

One of the treatment strategies for diabetes mellitus is to inhibit specific digestive enzymes in order to reduce carbohydrates absorption in the intestines. In this study, inhibitory activity toward the α-amylase enzyme were determined for the methanolic extracts of the leaves of two species of the genus *Crataegus*; *monogyna*, and *azarolus var aronia*, and three species of the genus *Rubus*; *sanctus*, *hedycarpus*, and *collinus.* The leaves were collected during spring and autumn seasons from different regions across the Syrian Arab Republic. The methanolic extract of *C.azarolus var aronia* harvested in autumn exhibited the strongest inhibitory activity toward α-amylase compared to the other extracts with an IC_{50}=103 µg/ml. For a closer look into this plant, the dry methanolic extract was suspended in water and extracted (liquid-liquid) using diethyl ether and ethyl acetate as extracting solvents. Due to the promising inhibitory activity of the ethyl acetate extract toward α-amylase with an IC_{50}=96 µg/ml, this extract was further fractionated by means of preparative HPLC. The structure of the isolated compound that revealed the strongest inhibitory activity toward α-amylase enzyme (IC_{50}=84 µg/ml) was elucidated using nuclear magnetic resonance (NMR), and mass spectroscopy (MS) techniques. The structure was identified based on MS and NMR data to be (–)-Epicatechin, a biologically active flavan-3-ol compound.

Key words: *Crataegus*; *Rubus*; α-amylase; MS; NMR; Epicatechin.

1. Introduction

Crataegus Known as hawthorn, is a plant widely spread in the northern hemisphere and is spread well in Syria as a wild plant. It has been used as food supplementary and traditional medicine for years. The genus name *Crataegus* is derived from the Greek word Kratos meaning hardness of the wood. *Crataegus* may be employed as anti-inflammatory, gastro-protective, antidiabetic, antimicrobial, and hepatoprotective. And it may improve coronary artery blood flow and heart muscle contractions, hence widely used as cardiac tonic [1–3].

Rubus Popularly known as the genus of raspberries and blackberries, is widely distributed in Syria, and worldwide as wild and cultivated species. The genus *Rubus*, with around 700 species, is the largest in the Rosaceae family. *Rubus* leaves demonstrate antioxidant, anticancer, antiangiogenic, antithrombotic, hypoglycemic, antimicrobial and anxiolytic activities [4–6].

Type 2 Diabetes mellitus is a metabolic disorder, characterized by defects in insulin secretion, insulin sensitivity, or both, promoting disturbance of carbohydrates, fat, and protein metabolism. Common complications of diabetes mellitus include retinopathy, nephropathy, neuropathy, micro-angiopathy, and increased risk of cardiovascular diseases. Delayed insulin secretion immediately after a meal leads to a sudden surge in blood glucose level known as 'hyperglycemic spikes'. The normal blood glucose level in two hours after meal is estimated to be not more than 139 mg/dl. This 2-hour postprandial blood glucose level will range from 140 to 199 mg/dl in the case of impaired glucose tolerance, and then rise to greater than 200 mg/dl in the case of diabetics. One of the anti-diabetic therapeutic strategies is the inhibition of carbohydrate digesting enzymes such as the α-amylase in order to reduce the absorption of sugars. α-amylase hydrolyzes complex starches to oligosaccharides. Inhibition of this enzyme reduces the rate and extent of glucose absorption produces a postprandial anti-hyperglycemic effect [7–10].

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The α–amylase (α-1,4-glucan-4-glucanohydrolases; E.C.3.2.1.1) is the major secretory products of the pancreas and salivary glands for digestion of starch and glycogen. The α-amylase catalyzes the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds. Oligosaccharides are the end products of α-amylase action. A variety of plant extracts revealed inhibition of α-amylase activities and could be relevant for the treatment of type 2 diabetes [11–14].

2. Experimental:

2.1. Materials:

Methanol (Fluka chemika), Distilled water, Ethyl acetate (Merck), Diethyl ether (Merck), Dimethyl sulfoxide (DMSO) (Merck), Starch, Acarbose (Tokyo chemical industry), α-amylase from porcine pancreas (Sigma-Aldrich), Dinitro salicylic acid (Sigma-Aldrich).

2.2. Equipments:

The plant's leaves were softened by a grinder. The absorbance was measured using a UV/Vis Spectrophotometer, type SP-3000 Plus OPTIMA TOKYO, JAPAN. The extracts were dried using a rotary evaporator, type Buchi Rotavapor R-205. The active extract was fractionated using HPLC semi preparative JASCO-LC-1500 using column C18 (5µm) 15*2.12cm. The Mass Spectrum was recorded using Shimadzu LC2020. The Nuclear Magnetic Resonance (NMR) spectra were recorded on Avance 400MHz Bruker, Germany.

2.3. Collection of plant material:

Leaves of 3 species of *Rubus* (*sanctus*, *hedycarpus*, and *collinus*) and 2 species of *Crataegus* (*monogyna* and *azarolus var aronia*) were collected in spring (April) and in autumn (October) from several areas across the Syrian Arab Republic in the early morning. The collected leaves were cleaned, dried, cut into small pieces, grounded, and sifted into a homogeneous powder. The resulting powders were stored separately until the extraction was carried out.

2.4. Extraction:

Methanolic extracts of each collected plant (leaves) were prepared according to the following steps: 50 grams of dry leaves powder was weighed and extracted by maceration in 500 ml methanol for 72 hours. The process was repeated three times. The extracts were filtered and dried using a rotary evaporator. A series of concentrations was prepared for each extract (25-50-100-200-400) µg/ml using DMSO 10% as a solvent.

2.5. Determination of α-amylase inhibitory activity:

0.5 ml of extract and 0.5 ml of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution (0.5 mg/ml) was placed in a tube. This tube was incubated at 25 C° for 10 min, after that 0.5 ml of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to the tube and further incubated for 10 min. 1ml of dinitrosalicylic acid (DNS) reagent (10mg/ml) was added to terminate the reaction. The tubes were then incubated at 100 C° for 5 min. and cooled to room temperature. The absorbance was measured at 540 nm using a spectrophotometer. Blank was prepared using the same procedure, replacing the extract by DMSO 10%. Triplicates are done for each extract. The α-amylase inhibitory activity was calculated as percentage inhibition.

$$(I\ \%) = 100 - ((A_{sample} - A_{sampleB}) / (A_{control} - A_{controlB}) \times 100)$$

Where (I%): α-amylase Inhibition%, A_{sample}: absorbance of the sample, $A_{sampleB}$: absorbance of the sample's blank, $A_{control}$: absorbance of the control, $A_{controlB}$: absorbance of the control's blank.

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically [10,15].

2.6. Isolation and structure elucidation of active compound:

2.6.1. HPLC preparative:

Separation was performed using a C18 preparative column 150*21.2 mm*5µm, applying a mobile phase consisted of: solvent A: Water, and solvent B: Acetonitrile (ACN). Gradient elution program was used applying a flow rate 2 ml/min as follows: Solvent B 10% for 5 min, B 10% to 30% in 5-8 min, B 30% to 100% in 8-30 min, B 100% to 10% in 30-37 min. Data were collected using UV detector at 254 nm

2.6.2. Mass spectroscopy analysis:

Conditions for mass spectral analysis in the Electrospray ionization (ESI) positive mode include a capillary voltage of 3500 v, drying gas temperature at 230 C°, gas flow 10L/min, nebulizer 50 psi.

2.6.3. NMR spectroscopy:

^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were measured and recorded in deuterated DMSO by means of an Avance 400MHz spectrometer. Chemical shifts (δ) were recorded in

parts per million (ppm) relative to the tetramethylsilane (TMS) signal used as internal standard. The signals were illustrated in terms of chemical shift with proper abbreviations for multiplicities and are reported as s (singlet), d (doublet), and t (triplet), Moreover, ^{13}C-Nuclear Magnetic Resonance (^{13}C-NMR) with off resonance decoupling heteronuclear multiple bond coherence (HMBC), heteronuclear multiple quantum coherence (HMQC), distortion less enhancement by polarization transfer (DEPT), and correlation spectroscopy (COSY) were all recorded on the Avance 400MHz spectrometer.

3. Results:

The average weight of the obtained dry extract ranged from 1.1 g to 3.3g (Table 1)

The biological study of the ability of the extracts to inhibit the α-amylase enzyme was performed. The IC_{50} values were calculated and compared for all samples to acarbose which was used as positive control. The IC_{50} values of the samples ranged from 103 to 231 µg / ml (Table 1).

Table 1. The average weight (g) of the obtained dry extract of the dried extracts and IC_{50} values (µg/ml) for α-amylase inhibition

Samples	*C.azarolus*		*C.monogyna*		*R.collinus*		*R.hedycarpus*		*R.sanctus*	
Season	au	sp	au	sp	au	sp	au	sp	au	sp
Average weight (g) of the obtained dry extract	3.3	2.7	2.4	1.9	2.2	1.6	3	2.5	1.9	1.1
α-amylase Inhibition IC_{50} µg/ml	103	119	125	148	178	231	190	217	112	162

3.1. Fractionation and isolation:

The methanolic extract of *C.azarolus* au, revealed the strongest inhibitory activity toward the α-amylase enzyme, was partitioned successively to obtain 3 fractions using diethyl ether (fraction A), ethyl acetate (fraction B), and water (fraction C). The 3 fractions were dried using a rotary evaporator and were tested for their α-amylase inhibition activity (Table 2).

Table 2. IC_{50} values (µg/ml) for α-amylase inhibition of fractions from *C.azarolus* au

Sample	*C.azarolus* au		
Fractions	FRACTION A	FRACTION B	FRACTION C
α-amylase Inhibition IC_{50} µg/ml	189	96	237

Fraction B (ethyl acetate fraction) that showed the most inhibitory activity toward the α-amylase enzyme was subjected to prepared HPLC. Two main peaks were observed in the spectrum. The two compounds (Cpd.1 and Cpd.2) under peak 1 and peak 2, with retention time of 16 and 18.5, respectively, were isolated and evaluated for their inhibitory activity toward α-amylase (Table 3).

Table 3. IC_{50} values (µg/ml) for α-amylase inhibition of isolated peaks

Sample	*C.azarolus* au (FRACTION B)	
Isolated compounds	Cpd.1	Cpd.2
α-amylase Inhibition IC_{50} µg/ml	136	84

Cpd. 2 exhibited a higher activity than Cpd. 1 with an IC_{50} value of 84 µg/ml. Therefore, a further investigation of Cpd. 2 was carried on, in order to elucidate its chemical structure. The structure was elucidated using Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS) techniques.

3.2. Spectral data of Cpd. 2:

ESI-MS (m/z): 291.1 $[M+H]^+$; **^{1}H-NMR** (DMSO-*d6*, δ [ppm], *J* [Hz]): 9.11 (S, 1H, -OH), 8.89 (S, 1H, -OH), 8.79 (S, 1H, -OH), 8.72 (S, 1H, -OH), 6.88 (d, *J* = 1.2, 1H, H2'), 6.65 (m, 2H, H-5', 6'), 5.88 (d, *J* = 2.3, 1H, H-6), 5.71 (d, *J* = 2.3, 1H, H-8), 4.73 (S, 1H, H2), 4.66 (d, *J* = 4.6, 1H, 3-OH), 4 (*brd*, *J* = 3.5, H-3), 2.67 (dd, 1H, *J* = 16.4, 4.4, H-4a), 2.46 (m, 1H, H-4b). **^{13}C-NMR** (DMSO-*d6*, δ [ppm]): 156.49, 156.21, 155.74, 144.47, 144.41, 130.58, 117.92, 114.87, 114.73, 98.74, 95.06, 94.07, 78.03, 64.89, 28.15.

3.3. Structure elucidation of Cpd. 2:

The NMR- and MS- spectroscopy analysis of the most active isolated substance (Cpd. 2) revealed a

skeleton of a flavon-3ol and was assigned to Epicatechin. The compound was isolated as yellow amorphous powder by means of preparative HPLC. The ESI-MS analysis shows a molecular peak at m/z: 291.1 $[M+H]^+$, which indicates molecular mass of 290 g/mol corresponding to the molecular formula of $C_{15}H_{14}O_6$ (Fig. 1.). The ^{13}C-NMR spectrum indicates 15 carbon atoms in the molecule. The DEPT spectrum indicates 7 methine groups, one methylene carbon at δ 28.15 ppm (assigned to carbon 4) and 7 quaternary carbon atoms. The ^{1}H-NMR spectrum exhibited 5 aromatic protons. The *meta*-coupling and the positioning of the aromatic protons δ 5.88 ppm and δ 5.71 ppm at positions C6 and C8, respectively, was confirmed by a coupling constant value of 2.3Hz and the correlations in the HMBC spectrum. The ^{1}H-NMR spectrum revealed two oxygenated methine groups at δ 4.73 ppm and δ 4.00 ppm. These signals were assigned to the protons at positions C2 and C3 respectively. The HMQC spectrum exhibits a correlation between the proton signal at δ 4.73 ppm to the carbon signal at δ 78.03 ppm (C2) and a correlation between the proton signal at δ 4.00 ppm to the carbon signal δ 64.89 ppm (C3). Two double doublets signals at δ 2.67 and δ 2.46 ppm are correlated to one carbon signal δ 28.15 ppm. HMQC indicated a methylene group at C4. The aromatic ring B is substituted with 3 hydrogens having the aromatic proton signals at δ 6.65 ppm for the positions C5` and C6` and at δ 6.88 ppm for C2`. The signal at δ 6.88 ppm shows a broad doublet with a coupling constant of 1.2Hz indicating a *meta*-coupling between the protons at positions C2` and C6`. The COSY spectrum exhibits correlations between the proton signal at δ 4.00 ppm (C3) to the signals at δ 4.73 ppm (C2), δ 2.67 ppm and at δ 2.64 ppm (C4). The HMBC spectrum shows correlations between the proton signal at δ 4.00 ppm to the carbon signals at δ 98.47 ppm (C10) and δ 13.58 ppm (C1`). A correlation between the protons of the methylene group at position 4 to the carbons C2, C5, C3 and C10 is to be seen in the HMBC spectrum. This information proves the binding of the benzene ring B to C2 and the distribution of the protons of the ring C. These results were similar to those reported in the literature [16–18].

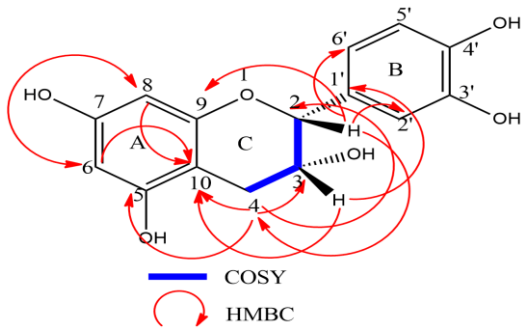

Fig. 1. HMBC and COSY correlations of Epicatechin

4. Discussion:

The inhibitory activity of α-amylase was measured for all extracts and compared to acarbose. The extract of *C.azarolus* au revealed the strongest inhibitory activity (IC_{50} = 103µg / ml), followed by the extract of *R.sanctus* au (IC_{50} = 112 µg/ml), while the extract *R.collinus* sp showed the lowest inhibitory activity (IC_{50} = 231 µg / ml). The control (Acarbose) exhibited an IC_{50} value of (61 µg / ml) (Table 1). A study conducted in Iran on a methanolic extract of *Crataegus oxyacantha*, and *Rubus fruticous*, revealed IC_{50} values of 62 µg/ml and 53.7 µg/ml respectively [19]. In another study in Tunisia on an aqueous extract of *Crataegus azarolus var aronia*, it was found that the IC_{50} ranged between 1810-3010 µg/ml [20]. A study conducted in Nepal on a methanolic extract of *Rubus ellipticus*, reported an IC_{50} value of 296.94 µg/ml [21]. And it was observed that the extracts obtained in autumn of each species revealed a higher inhibitory activity toward α-amylase enzyme than the extracts obtained in spring.

Most of the plant related biological activities are due to the presence of secondary plant metabolites such as Phenolics and flavonoids [22,23]. Several studies have indicated the effect of the harvesting date on the chemical content of the plant, especially for the polyphenol products [24,25]. The concentration of the polyphenols in the plants including flavan-3-ols is known to be higher during summer and autumn compared to spring, resulting in a stronger biological activity for the plants collected in these seasons. That supports results obtained in this study, especially that the most active compound (-)-Epicatechin is a polyphenol metabolite (flavan-3-ol) [24–27].

5. Conclusion:

Crataegus and *Rubus* extracts exhibited a potential inhibitory activity toward the α-amylase enzyme. Epicatechin, a flavan-3-ol compound, isolated from *C.azarolus var aronia* extract, was found to be the most active compound in the extract toward the α-amylase enzyme. The isolation of the epicatechin was carried out by using a chromatographic separation and its identity was confirmed by MS- and NMR- spectroscopy.

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8. Arabic Abstract:

تقصي الفعالية المثبطة لأنزيم الألفاأميلاز لبعض أنواع الزعرور والعليق المنتشرة في سوريا، وعزل وتحديد بنية المركب الفعال

يعتبر تثبيط بعض الأنزيمات الهاضمة المسؤولة عن هضم السكريات، أحد الاستراتيجيات المتبعة لعلاج داء السكري، وذلك بهدف تقليل امتصاص السكريات في الأمعاء. في هذه الدراسة، تم تحديد النشاط المثبط لأنزيم الألفاأميلاز للخلاصات الميتانولية لنوعين من جنس الزعرور هي *Crataegus monogyna*، و *Crataegus azarolus var aronia*، ولثلاثة أنواع من جنس العليق هي *Rubus sanctus*، و *Rubus hedycarpus*، و *Rubus collinus* المجنية في كل من فصلي الربيع والخريف من مناطق مختلفة في الجمهورية العربية السورية. أظهرت الخلاصة الميتانولية للنوع *C.azarolus var aronia* المجنية في فصل الخريف أعلى فعالية مثبطة لأنزيم الألفاأميلاز مقارنة بالخلاصات الأخرى، حيث كانت IC50=103 مكغ\مل. وللتحري عن المركب الفعال تم تجزئة الخلاصة الميتانولية إلى ثلاثة خلاصات باستخدام كل من الماء، وخلات الإيتيل، والإيتر كمحلات. ثم تم تجزئة خلاصة خلات الإيتيل والتي أعطت أعلى فعالية مثبطة لأنزيم الألفاأميلاز IC50=96 مكغ\مل بواسطة جهاز الكروماتوغرافيا التحضيرية HPLC-Preparative. تم تحديد بنية المركب المعزول الذي أظهر أعلى فعالية مثبطة لأنزيم الألفاأميلاز IC50=84 مكغ\مل باستخدام تقنيات مطياف الكتلة MS والرنين النووي المغناطيسي NMR ليكون هو الإيبيكاتيشين Epicatechin-(–) وهو مركب فلافان-3-ول الذي يملك فعالية حيوية.