

# ASSESSMENT OF IN-VITRO ANTIDIABETIC & ANTIOXIDANT EFFECTS OF *Helianthus tuberosus*, *Cydonia oblonga* AND *Allium porrum*

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## Abstract

*In-vivo* antidiabetic activity of *Cydonia oblonga* Mill. (quince) leaves, *Helianthus tuberosus* L. (Jerusalem artichoke) tubers, and *Allium porrum* L. (leek) bulb and leaves was determined in our previous studies. These plants are traditionally used to lower blood glucose levels in Turkey. Therefore, inhibitory activities of quince leaves, Jerusalem artichoke tubers, leek bulbs and leaves on carbohydrate digestive enzymes ( $\alpha$ -glucosidase and  $\alpha$ -amylase) were investigated to enlighten the mechanism of action. On the other hand, their antioxidant activities, total phenol and flavonoid contents were investigated. The highest ABTS radical scavenging, total antioxidant activity and reducing power were found in *C. oblonga* leaf ethanol extract. Nevertheless, tested plant extracts did not inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase significantly. Total phenol and flavonoid contents of the extracts were found in the range of 7.91-163.33 mg gallic acid equivalent/g and 27.26-29.60 mg quercetin/g, respectively. Consequently, it is clearly seen that the mechanism of action of these three plants was not determined to be related to the inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.

**Keywords:** Antioxidant, *Allium porrum*, *Cydonia oblonga*, *Helianthus tuberosus*, Hypoglycemic

## *Helianthus tuberosus*, *Cydonia oblonga* ve *Allium porrum*'un *In Vitro* Antidiabetik & Antioksidan Etkilerinin Değerlendirilmesi

*Cydonia oblonga* Mill. (ayva) yaprakları, *Helianthus tuberosus* L. (yer elması) yumruları ve *Allium porrum* L. (pırasa) soğan ve yapraklarının *in-vivo* antidiabetik etkileri daha önceki çalışmamızda tespit edilmiştir. Bu bitkiler geleneksel olarak kan glukoz seviyelerini düşürmek için kullanılmaktadır. Bu sebeple, etki mekanizmalarını aydınlatmak için; ayva yaprakları, yer elması yumruları, pırasa soğan ve yapraklarının karbonhidrat sindiren enzimler ( $\alpha$ -glukozidaz ve  $\alpha$ -amilaz) üzerindeki inhibitör etkileri incelenmiştir. Diğer yandan, antioksidan aktiviteleri, total fenol ve flavonoid içerikleri incelenmiştir. En yüksek ABTS süpürücü aktivite, total antioksidan aktivite ve indirgeyici güç, *C. oblonga* yaprak etanol ekstresinde bulunmuştur. Ekstrelerin, total fenol ve flavonoid içerikleri sırasıyla 7.91-163.33 mg gallik asit eşdeğer/g ve 27.26-29.60 mg kersetin/g aralığında bulunmuştur. Sonuç olarak, bu üç bitkinin etki mekanizmasının  $\alpha$ -amilaz ve  $\alpha$ -glukozidaz enzimlerinin inhibisyonu ile ilgili olmadığı tespit edilmiştir.

**Anahtar kelimeler:** Antioksidan, *Allium porrum*, *Cydonia oblonga*, *Helianthus tuberosus*, Hipoglisemik

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## INTRODUCTION

Diabetes mellitus is a metabolic disease induced by deficiency or diminished effectiveness of insulin. The chronic hyperglycemia causes to generation of reactive oxygen species (ROS) playing a serious role in the occurrence of organ injuries in diabetes. Antioxidant defense system of organism plays a crucial role to improve deleterious effects of ROS (1). Therefore, consumption of dietary antioxidants is essential to prevention of tissue and organ damages caused by uncontrolled production of ROS in diabetes.

In different localities of Anatolia, *Allium porrum* L. (Liliaceae) is used in the treatment of hyperlipidemia, infertility, hemorrhoids. Additionally, the fresh bulbs of the plant known as leek are boiled and drunk (1-2 glasses) before meals against diabetes in rural area (2-5). Tubers of *Helianthus tuberosus* L. (Asteraceae), are utilized as a diuretic, lactagogue, aphrodisiac, antihemorrhoidal and collagogue and fresh tubers of the plant are consumed to decrease diabetes symptoms (4, 6, 7). On the other hand, the leaf decoction of *Cydonia oblonga* Mill. (Rosaceae) has been used traditionally in the treatment of cough, cold, bronchitis, abdominal pain, diarrhoea, disurea, and against hyperglycemia in Turkey (2-4, 8).

In our previous study, the antidiabetic activities of these three species have been observed on streptozotocin induced diabetic rats (9). Thus, we aimed to enlighten the mechanism of action by evaluating *in vitro* antidiabetic activities of these plants. Additionally, antioxidant activities of these plants were determined and also total phenol and flavonoid contents were measured.

## EXPERIMENTAL

### *Plant materials and extraction*

Fresh *Cydonia oblonga* leaves were collected from Ankara (September, 2014), *Allium porrum* bulbs and *Helianthus tuberosus* tubers were purchased from bazaar (February, 2015). The plant materials were shredded using ultra-torax. *H. tuberosus* (500 g), *A. porrum* (500 g), and *C. oblonga* (50 g) were extracted separately with 80% aqueous EtOH (2 L × 2 times) for 6 hours in a 50 °C water bath. Each extract was filtered and concentrated until dryness under reduced pressure in a rotary evaporator. Yields of the extracts were 24.1 % for *C. oblonga* (CO), 3.5 % for *A. porrum* (AP), and 12.3% (w/w) for *H. tuberosus* (HT).

### *Assay for $\alpha$ -glucosidase inhibitory activity*

*Bacillus stearothermophilus* originated  $\alpha$ -Glucosidase type IV enzyme (Sigma Co., St. Louis, USA) was dissolved in 0.5 M phosphate buffer (pH 6.5). The enzyme solution and test extracts dissolved in ethanol were preincubated in a 96-well microtiter plate for 15 min at 37 °C. After that, the substrate solution [20 mM *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (NPG), Sigma] in the same buffer was added. The mixture was incubated for 35 min at 37 °C. The increase in the absorption at 405 nm due to the hydrolysis of NPG by  $\alpha$ -glucosidase was measured by a ELISA microtiter plate reader (10). Acarbose (Bayer Group, Turkey) was used as positive control. The inhibition percentage (%) was calculated by the equation:

$$\text{Inhibition \%} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

### *Assay for $\alpha$ -amylase inhibitory activity*

The  $\alpha$ -amylase inhibitory activity of the extracts was determined by the chromogenic method of Ali et al. (11). Porcine pancreatic  $\alpha$ -amylase (EC 3.2.1.1, type VI, Sigma) was dissolved in distilled water. As substrate solution, potato starch (0.5 %, w/v) in phosphate buffer (pH 6.9) was used.

Plant extracts and distilled water were mixed in a tube and the reaction was initiated by the addition of the enzyme solution. Then the tubes were incubated at for 3 min. Starch were added and the tubes were incubated for 5 min. After that, DNS colour reagent solution was added to the mixture and put into a 85 °C heater. After 15 min, distilled water was added to the tubes and tubes were cooled. Absorbances of the mixtures were read at 540 nm. Acarbose was used as the positive control. The absorbance (A) due to maltose generated was calculated according to following formula:  $A_{\text{control or plant extract}} = A_{\text{Test}} - A_{\text{Blank}}$

The amount of maltose generated was calculated by using the maltose standard calibration curve (0 - 0.1% w/v) and the obtained net absorbance. Percent of inhibition was calculated as:

$$\text{Inhibition \%} = [(Maltose_{\text{control}} - Maltose_{\text{sample}}) / Maltose_{\text{control}}] \times 100$$

#### *Estimation of DPPH radical scavenging activity*

The DPPH radical scavenging activity of the extracts was determined in a 96 well-plate (12). 160  $\mu\text{L}$  of extract was mixed with 40  $\mu\text{L}$  of DPPH solution and incubated in darkness for 30 min. Then the absorbance was measured at 520 nm utilizing a 96-well ELISA microplate reader (VersaMax, Molecular Devices, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Ascorbic acid was used as a positive control at 0.57, 1 and 3 mg/mL concentrations.

#### *Metal chelating activity*

Extracts were incubated with  $\text{FeCl}_2$  (2 mM). The reaction was initiated by the addition of 0.2 mL of ferrozine (5 mM) and the total volume was adjusted to 4 mL with ethanol. After 10 min, the absorbance was measured at 562 nm. EDTA was used as a reference compound. The percentage of inhibition of the ferrozine- $\text{Fe}^{+2}$  complex formation was calculated using this formula: Metal chelating activity (%) =  $[(A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}}] \times 100$ . The control contained  $\text{FeCl}_2$  and ferrozine (13).

#### *Ferric-reducing antioxidant power*

Different logarithmic concentrations of the extracts (3, 1, and 0.57 mg/mL) and ascorbic acid as reference were mixed with phosphate buffer (0.2 mol/l, pH 6.6) and  $\text{K}_3\text{Fe}(\text{CN})_6$ . Tubes were incubated at 50 °C for 20 min, then trichloro acetic acid was added and the mixture was vortexed. Following centrifugation, a part of the supernatant was mixed with distilled water and  $\text{FeCl}_3$  and the absorbance at 700 nm was measured (14).

#### *Total antioxidant activity by phosphomolybdenum assay*

This test is generally based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH. Extracts were added to test tubes containing distilled water and molybdate reagent solution. Vortexed tubes were incubated at 90 °C for 90 min. Then, tubes were cooled to room temperature and the absorbances of the samples were measured at 695 nm. Results were expressed as ascorbic acid equivalent (15). BHT and BHA were used as reference.

#### *Assay for scavenging activity of ABTS radical cation*

ABTS radical cation ( $\text{ABTS}^+$ ) scavenging assay was achieved by using the spectrophotometric methods of Re et al. (16) and Meot-Duros et al. (17) with slight modifications. ABTS (7 mM) was dissolved in distilled water and the ABTS radical cation was generated by adding 2.45 mM potassium per-sulfate. The radical production was completed after incubation for 16 hour in the dark at 20°C. Absorbance of ABTS solution was adjusted to  $0.7 \pm 0.02$  at 734 nm by the addition of phosphate buffer solution (PBS) at pH 7.4. 1 mL diluted ABTS solution was added to 10  $\mu\text{l}$  of extract (PBS or gallic acid). Samples were vortexed and

their absorbances were read versus PBS blank at 734 nm. Gallic acid was used as the positive control.

#### Determination of total phenol content

The extracts were mixed with Folin-Ciocalteu reagent (10%) and samples were incubated for 5 min. at room temperature. Then, sodium carbonate solution (7.5 %) was added and samples were vortexed immediately. The absorbance of mixture was measured at 735 nm after 30 min. at room temperature in a dark place. The mean of three readings was used and the total phenol content was expressed in mg of gallic acid equivalents (GAE)/g extracts (18). Calibration curve equation was;  $y(\text{Abs.})=5.306x(\text{Conc.})+0.0587$  and the coefficient of determination was  $r^2=0.9986$ .

#### Determination of total flavonoid content

Dry extracts were dissolved in 80% ethanol. 95% ethanol, 1 M sodium acetate and aluminum chloride solution (10%) were added to the samples and the mixture was diluted to 5 mL by distilled water. After 30 min. incubation at room temperature, the absorbance of yellow mixtures was measured at 415 nm. Methanol was used as blank. Results were expressed in mg of quercetin equivalents (QE)/g extracts (19). Calibration curve equation was;  $y(\text{Abs.})=2.4214x(\text{Conc.})-0.051$  and the coefficient of determination was  $r^2=0.9998$ .

#### Statistical analysis

All experiments were carried out with minimum three replicates. Values were presented as means  $\pm$  standard error of the mean (S.E.M.) or standard deviation (S.D.). Linear regression analyses were done using MS-DOS software (GraphPad InStat statistical program). Inhibition and change percentages were calculated as following:

$$\text{Inhibition/Change \%} = (\text{Test Value}/\text{Control Value}) \times 100 - 100$$

## RESULTS AND DISCUSSION

As seen in Table 1,  $\alpha$ -glucosidase inhibitory activity of ethanol extracts of tested plants is in the order as CO leaf >HT tuber >AP bulb & leaves. The maximum inhibition values of all extracts varied between 10.87% and 16.37%. In this enzyme model, acarbose showed 97.13% inhibition at 100  $\mu\text{g}/\text{mL}$ . Among the tested extracts, only HT tuber ethanol extract exhibited very low  $\alpha$ -amylase inhibitory activity (0.49%) when compared to acarbose (64.50 %) at 1000  $\mu\text{g}/\text{mL}$ . However, CO leaf and AP bulb & leaf extracts failed to inhibit  $\alpha$ -amylase enzyme.

**Table 1.**  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activity of ethanol extracts

Plant Name	Plant Part	$\alpha$ -Glucosidase Inhibitory Activity (Inhibition % $\pm$ S.D.)				$\alpha$ -Amylase Inhibitory Activity (Inhibition % $\pm$ S.D.)	
		3000 $\mu\text{g}/\text{mL}$				3000 $\mu\text{g}/\text{mL}$	
AP	Bulb & Leaves	10.87 $\pm$ 1.12				X	
CO	Leaves	16.37 $\pm$ 0.89				X	
HT	Tubers	13.60 $\pm$ 2.54				0.49 $\pm$ 0.03	
Acarbose	Concentration	100 $\mu\text{g}/\text{mL}$	30 $\mu\text{g}/\text{mL}$	10 $\mu\text{g}/\text{mL}$	1000 $\mu\text{g}/\text{mL}$	300 $\mu\text{g}/\text{mL}$	100 $\mu\text{g}/\text{mL}$
	Inh. % $\pm$ S.D.	97.13 $\pm$ 0.24	95.02 $\pm$ 0.32	90.87 $\pm$ 0.42	64.50 $\pm$ 1.72	38.06 $\pm$ 1.00	16.40 $\pm$ 1.68

AP: *Allium porrum*, CO: *Cydonia oblonga*, HT: *Helianthus tuberosus*, X: No Activity

Total antioxidant assay results showed that CO leaf extract has the highest antioxidant capacity ( $126.84 \pm 3.31$  AAE) followed by AP bulb & leaf extract ( $38.35 \pm 1.82$  AAE) (Table 2). Total antioxidant capacity of CO leaf extract was found higher than or close to those of BHT ( $54.74 \pm 8.67$  AAE) and BHA ( $159.62 \pm 3.28$  AAE) used as reference antioxidant compounds. On the other hand, we did not observe any antioxidant activity in HT tuber extract at 3000  $\mu\text{g/mL}$  concentration in phosphomolybdenum assay.

Among the tested extracts, CO leaf extract at 3000  $\mu\text{g/mL}$  concentration displayed the highest ABTS cation radical scavenging activity (81.40%), while HT tuber extract showed no activity. In this assay, ABTS cation scavenging activity of reference compound gallic acid ranged from 90.87 to 97.13% at tested concentrations (Table 2).

**Table 2.** Total antioxidant activities, ABTS and DPPH radical scavenging activities of ethanol extracts

Plant Name/ Reference	Total Antioxidant (AAE $\pm$ S.D.)	ABTS Cation Radical Scavenging Activity (Inhibition % $\pm$ S.D.)			DPPH Radical Scavenging Activity (Inhibition % $\pm$ S.D.)		
		3000 $\mu\text{g/mL}$	1000 $\mu\text{g/mL}$	570 $\mu\text{g/mL}$	3000 $\mu\text{g/mL}$	1000 $\mu\text{g/mL}$	570 $\mu\text{g/mL}$
AP	$38.35 \pm 1.82$	$3.71 \pm 0.28$	$3.17 \pm 0.01$	$1.65 \pm 0.23$	$47.99 \pm 0.54$	$16.41 \pm 1.93$	$13.48 \pm 0.91$
CO	$126.84 \pm 3.31$	$81.40 \pm 1.56$	$31.08 \pm 0.68$	$15.93 \pm 0.29$	$24.10 \pm 2.13$	$30.38 \pm 2.25$	$29.97 \pm 1.13$
HT	X	X	$20.25 \pm 4.97$	$1.38 \pm 0.58$	X	$13.58 \pm 2.54$	$18.24 \pm 1.80$
BHT	$54.74 \pm 8.67$	NT	NT	NT	NT	NT	NT
BHA	$159.62 \pm 3.28$	NT	NT	NT	NT	NT	NT
Ascorbic A.		NT	NT	NT	$72.88 \pm 1.62$	$75.27 \pm 1.66$	$69.62 \pm 5.68$
Gallic Acid		1000 $\mu\text{g/mL}$	570 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$			
	NT	$99.85 \pm 0.00$	$99.30 \pm 0.17$	$98.94 \pm 0.30$	NT	NT	NT

AP: *Allium porrum*, CO: *Cydonia oblonga*, HT: *Helianthus tuberosus*, X: No Activity, NT: Not Tested

DPPH radical scavenging capacity of the extracts was found to be lower than that of ascorbic acid (75.27%). The highest DPPH scavenging activity was detected in AP bulb & leaf extract with 47.99%. Interestingly, as seen in Table 2, DPPH radical scavenging activity decreased when the concentration of HT and CO ethanol extracts were increased.

The reducing capacity of CO leaf extract ( $0.1686 \pm 0.0016$ ) was close to that of ascorbic acid ( $0.1868 \pm 0.0016$ ) at 3000  $\mu\text{g/mL}$  concentration (Table 3). HT extract exhibited the lowest reducing power, with an absorbance of 0.0030 at the concentration of 3000  $\mu\text{g/mL}$ .

**Table 3.** Reducing power and metal-chelating capacity of ethanol extracts

Plant Name/ Reference	Reducing Power (Absorbance $\pm$ S.D.)			Metal Chelating Capacity (Inhibition % $\pm$ S.D.)		
	3000 $\mu\text{g/mL}$	1000 $\mu\text{g/mL}$	570 $\mu\text{g/mL}$	3000 $\mu\text{g/mL}$	1000 $\mu\text{g/mL}$	570 $\mu\text{g/mL}$
AP	$0.0566 \pm 0.0013$	$0.0172 \pm 0.0010$	$0.0085 \pm 0.0002$	>100	$92.59 \pm 0.54$	$92.36 \pm 1.91$
CO	$0.1686 \pm 0.0016$	$0.1487 \pm 0.0017$	$0.1200 \pm 0.0022$	>100	$73.67 \pm 3.60$	$33.12 \pm 3.73$
HT	$0.0030 \pm 0.0010$	$0.0038 \pm 0.0001$	$0.0089 \pm 0.0003$	>100	$95.12 \pm 1.33$	$94.27 \pm 2.33$
EDTA	NT	NT	NT	>100	$96.18 \pm 1.20$	$95.83 \pm 1.48$
Ascorbic Acid	$0.1868 \pm 0.0016$	$0.1845 \pm 0.0075$	$0.181 \pm 0.0084$	NT	NT	NT

AP: *Allium porrum*, CO: *Cydonia oblonga*, HT: *Helianthus tuberosus*, NT: Not Tested

The iron chelating activity of all ethanol extracts (100%>) was found as much as that of EDTA (100%>) at 3000 µg/mL concentration (Table 3). However, CO extract at 570 µg/mL concentration (33.12%) showed the lowest iron chelating activity.

It is surprising that HT tuber extract (at 3000 µg/mL) did not exhibit any ABTS and DPPH radical scavenging activity. Additionally, its total antioxidant capacity could not be determined. We thought that these negative results could be related to the solubilization problem that there were some precipitates in this concentration of the HT extract.

The total phenol content in the extracts showed different values in the range of 7.91-163.33 mg GAE/g. CO ethanol extract had the highest total phenol content (163.33 mg GAE/g) and the lowest for HT ethanol extract (7.91 mg GAE/g). On the other hand, total flavonoid amount of all ethanol extracts was determined to be approximately similar and varied from 27.26 to 29.60 mg/g expressed as quercetin equivalent (Table 4).

**Table 4.** Total flavonoid and total phenol contents of ethanol extracts

Plant Name	Plant Part	Total Flavonoid Content (mg QE/g ± S.D.)	Total Phenol Content (mg GAE/g ± S.D.)
AP	Bulb & Leaves	27.81 ± 3.93	10.23 ± 1.36
CO	Leaves	27.26 ± 0.41	163.33 ± 6.05
HT	Tubers	29.60 ± 5.23	7.91 ± 1.62

AP: *Allium porrum*, CO: *Cydonia oblonga*, HT: *Helianthus tuberosus*

In our previous study, *in-vivo* antidiabetic and antioxidant activities of the ethanol extracts of AP, CO and HT have been studied in normal and streptozotocin-induced diabetic rats. All extracts have been administrated orally to rats at the doses of 250 and 500 mg/kg. In order to determine antioxidant activity, thiobarbituric acid reactive substance (TBARS) and reduced glutathione (GSH) levels in liver, kidney, and heart tissues have been measured by using spectrophotometric methods. CO (500 mg/kg) and AP (500 mg/kg) extracts have caused a decrease in blood glucose levels by 33.8% and 18.0%, respectively after 5 days administration to diabetic rats. Moreover, AP and CO extracts have induced significant alleviation on heart tissue TBARS levels (44.6 and 45.7%), HT and AP extracts have shown an inhibitory effect on kidney tissue TBARS levels (24.5 and 14.8%). These findings exhibited that all tested extracts have potent *in-vivo* antilipoperoxidant activity on oxidative stress caused by diabetes mellitus (9).

Thus, in this study we aimed to evaluate *in-vitro* antidiabetic and antioxidant activities of the same extracts. Our results are parallel to our previous results that  $\alpha$ -glucosidase inhibitory activity of CO and AP extracts was found higher than that of HT extract as in *in-vivo* experiments. Additionally, *in-vivo* antioxidant activities of the extracts are supported by *in-vitro* studies.

## CONCLUSION

Approaches to the control of blood glucose and prevention of hyperglycaemia are central to the treatment of diabetes mellitus. Appetite suppressants, inhibitors of digestion ( $\alpha$ -glucosidase inhibitors, guar gum, polysaccharides), insulin secretagogues, insulin potentiators, stimulants of glucose utilization, insulin mimetics, inhibitors of gluconeogenesis and glucogenolysis are used to balance blood glucose (20). We conducted this study to see if the antidiabetic activities of AP, CO and HT extracts are related to their carbohydrate digestive enzyme inhibitory effects or not. Consequently, the mechanism of action of these plants with potential antidiabetic properties

was not related to the inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes and they can expose their antidiabetic effects by other mechanisms on different pathways.

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