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In vitro α-Glucosidase and α-Amylase Enzyme Inhibitory Effects in *Elaeagnus angustifolia* Leaves Extracts

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ABSTRACT

This study was performed to demonstrate α-glucosidase and α-amylase enzyme inhibitor activities using the methanolic extracts of *Elaeagnus angustifolia* leaves. Methanolic extracts were prepared by two different extraction methods [accelerated solvent extractor (ASE) and Soxhlet apparatus]. The analytical procedure involved the application of liquid chromatography. Our results showed that the ASE extracts inhibited α-amylase (10-100 μg/ml) and α-glucosidase (10-50 μg/ml) dose-dependently exhibitedrp inhibitory activities with the extracts [α-amylase (40%) and α-glucosidase (56%)]. RP-HPLC analyses of the methanolic extracts were detected with vanillic acid and 4-hydroxybenzoic acid as the main phenolic acids in all the ASE extracts. *Elaeagnus angustifolia* leaf extracts may be attributed to the presence of vanillic acid and 4-hydroxybenzoic acid, there by confirming with its traditional use for the management of diabetes mellitus. We implicate that the anti-diabetic activity through in vitro assessments and support to the folkloric use of this plant for controlling type-2 diabetes mellitus in Turkey.

Keywords: *Elaeagnus angustifolia*, RP-HPLC, phenolic, ASE

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder which has a worldwide significance of defining as one of the five leading causes of deaths and affecting more than 100 million people. It disorders fat, protein and carbohydrate metabolisms, and is accompanied by many complications that are ordinarily irreversible vascular changes [1, 2]. Diabetes mellitus is classified into two types: type 1, also known as (insulin- dependent diabetes mellitus (IDDM) and type 2, (non- insulin-dependent diabetes mellitus (NIDDM) [2]. Type 2 DM proceeds from deficient insulin secretion, dysfunction of insulin or both [3]. Approximately 1.200 species of

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plants are used as folk medicines for the treatment of type-2 DM in the world [4-6]. In Turkey, it is reported that at least ten different plants are widely used for type-2 DM treatment. Some of them are prepared as tea, such as; black and green tea (*Camellia sinensis*), bergamote (*Citrus bergamia*), sage (*Salvia officinalis*), rosehip (*Rosa canina*), and cinnamon (*Cinnamomum cassia*) [7]. It has also been used to treat DM with a mixture of olive leaves and decoction of the leaves and branches of *Elaeagnus angustifolia* (Elaeagnaceae, Russian olive, oleaster) [8]. It is reported that the fruits of this plant are traditionally used as an appetizer in the East and Central Anatolia of Turkey. In addition, it has long been used to decrease the glucose rate in the blood by using decoction prepared with the leaves of the plant [9]. According to the peer-reviewed literature, soluble sugars and phenolic compounds (isorhamnetin, isorhamnetin-3-O-β-D-galactopryanoside and caffeic acid) have been isolated in oleaster fruits [10]. Tannins, flavones, carotenoids and polyphenolcarboxylic acids were identified in leaves and flavones, fats were isolated in theflowers of this plant [11]. Also, p-coumaric acid, in a larger quantity before hydrolysis and kaempferol, in a larger quantity after hydrolysis have been reported in soft extracts from young branches of *E. Angustifolia* [12]. Flavonoid compounds have been reported in *E. angustifolia* leaves also [13].

Glucosidase is one of the key enzymes that exhibitits effects by decreasing of the blood glucose level, hence the inhibition of this enzyme plays important role in the management of type-2 diabetes [14].

Many studies have reported that pancreatic α-amylase inhibitors from herbal drugs promise for diabetes mellitus treatment by exerting their bioactive compounds and enzyme inhibitory effects of the plants [15]. Managing DM by using carbohydrate digestive enzyme inhibitors from herbal drugs provides less adverse effects (e.g., liver problems, hypoglycemia at higher doses, etc.) than the synthetic drugs [15, 16].

Earlier studies have also reported that plant phenolics inhibit the activity of α-amylase enzyme and have ahypoglycemic effect [17]. In view of these reports, thepresent study aimed to investigate the anti-diabetic activity through in vitro assessments by the inhibition of α-glucosidase and α-amylase in *Elaeagnus angustifolia* leaves extract, which lead usto the suggested folkloric use of this plant for controlling type-2 diabetes mellitus in Turkey.

EXPERIMENTAL

Plant materials

Elaegnus angustifolia leaves were collected from the region around Kocaeli (Turkey), during the month of September, 2011. The plant material was identified reviewing the standard text "Flora of Turkey and the East Aegean Islands", Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey [18].

Chemicals/Reagents

Vanillic acid and ferrulic acid were purchased from Fluka(Steinheim, Germany). p-coumaric acid, caffeic acid and protocatechuic acid were supplied by Sigma-Aldrich (Steinheim, Germany). 4-hydroxy benzoic acid and acetonitrile were obtained from Merck (Darmstadt, Germany). 4-nitrophenyl-α-D-glucopyranoside (*p*NPG) (Sigma N1377), α-glucosidase (EC3.2.1.20), α-amylase (EC 3.2.1.1) (Sigma 10065), disodium hydrogenphosphate (Aldrich 431478), sodium carbonate (Sigma S7795) were used. Methanol and acetonitrile were obtained from Sigma (Ultrapure %99). All other reagents used were of analytical grade.

Extraction Method

Leaves of *Elaeagnus angustifolia* were air dried at room temperature in the shadow and powdered. They were extracted by two different methods and seven different samples. The sample (10 g) was extracted with methanol by Soxhlet apparatus (Supelco 64826), in 3 h. The solution was filtered and evaporated to dryness under reduced pressure to give 2.71 g methanol extract.

Soxhlet extraction plant material (10.13 g of *Elaeagnus angustifolia*) was placed in a 250 ml Soxhlet apparatus. The extraction wascarried out using methanol. The extraction continued for 3 h and evaporated to dryness (2.71 g) at 40 °C.

The plant materials weigh out 10.3 g; 10.25 g; 10.02 g; and 10.12 g, respectively. Samples were placed in the stainless steel extraction cell (11 ml) of a Dionex ASE 300 Accelerated Solvent Extractor (ASE) (Sunnyvale, CA, USA). Glass fibres filters were placed at the outlet of the extraction cell in order to prevent blocking up of the frit. Plant materials were extracted with methanol (100 %).

Extractions were performed in the following conditions: pressure-1.500 psi, temperature -50°C, set time 13 min, flush volume 60%, purge time 90 sec, and static cycle –1 and 2. The obtained extracts were concentrated under reduced pressure and transferred (after dissolving in small portions of solvents) to graduated flask (10 mL) [19].

HPLC Analysis

For polyphenol analysis, detection and quantification were carried out with Shimadzu CBM-20A (Kyoto, Japan), LC-10AT pump, CTO-10ASvp column oven, SIL-20AC HT Autosampler and SPD-M10Avp (Diode Array Detector. The ACE 5 C18 (250*4.6 mm) (Aberdeen, Scotland) column was used. The mobile phase consisted of H_2O/ACN (88/12; v/v) at a flow rate of 1.0 ml/min with isocratic elution andthe injection volume was 50 µl. The pH of the mobile phase containing 35 mM formic acid and adjusted to 4.30 by the addition of sodium hydroxide. Each extract was injected at least in triplicate [20, 21].

Assay for α-Glucosidase Inhibitory Activity

α-Glucosidase (40 µl) which is purified from Baker's yeast was mixed with plant extract samples (as an inhibitor) at various concentrations (10-25-50-100-250 µl). After incubation at 37°C for 10 min, 50 µl 4 mM pNPG (0.1 M, pH 6.9) was added to start the reaction. The reaction content was incubated again at 37°C for 30 min and then stopped by the addition of 1 ml 0.1 M Na_2CO_3 [22]. One Unit of enzyme activity is defined as the amount of α-glucosidase required to released one µmol of p-nitrophenol (PNP) from p-nitrophenyl-α-glucopyranoside per min. The released PNP from PNPG by the activation of α-glucosidase was determined by measuring the spectrum at 400 nm (Carry 60 UV-Vis, USA). The reaction mixture without the plant extract sample (i.e., no inhibitor) was used as a control. The experiment was performed in triplicate. Enzyme activities were estimated with the molar extinction coefficient 17.4 mM^{-1}/cm. The percent inhibition of α-glucosidase was calculated as [($Abs_{control}$− Abs_{sample} / $Abs_{control}$)× 100], where Abs_{sample} represents the absorbance of the experimental sample, $Abs_{control}$ represents the absorbance of the control. The α-glucosidase inhibitoryactivity was expressed as percentage intibition [23].

Assay for α-Amylase Inhibitory Activity

α-Amylase (400 µl) was mixed with the plant extract samples (as inhibitor) at varying concentrations (10-25-50 µl), incubated at room temperature and 0.5% starch solution was added as a substrate to start the reaction. Incubation at 37°C was carried out for 30 min and terminated by the addition of 500 µl 3,5-dinitrosalicylic acid (DNS) reagent and boiled at 100 °C for 15 min and diluted by the adding of 5 ml distilled water. The experiment was performed in triplicate. α-Amylase activity was measured at 540 nm (Carry 60 UV-Vis, USA). The controls without the plant extracts were also, assessed simultaneously, as a reference. The α-glucosidase inhibitory activity was expressed as percentage intibition using the following equation:

$$\text{Inhibition \%} = [(Abs_{control} − Abs_{sample} / Abs_{control})× 100] \tag{1}$$

RESULTS AND DISCUSSION

HPLC

In this study, the HPLC analyses for the quantitative determination of phenolic acid compounds (Compounds: 1-6) in Russian olive leaves were carried out by peak assignment of the retention times.

The chromatograms of the six phenolic acid standards and Russian olive (oleaster) methanolic leaf extract (by ASE 2/1) mentioned above is shown in **Figures 1 and 2**. The phenolic acid compounds determined by HPLC were eluted between 5.5 and 33 min. The detection limits (LODs), linearity of calibration which are obtained for this method, are presented in **Table 1**.

Table 1. Limit of detection (LOD), correlation coefficients (R^2), for HPLC

Compounds	LOD (mg/kg)	R^2
Protocatechuic acid	0.053	0.999
4-Hydroxy benzoic acid	0.036	0.999
Vanillic acid	0.070	0.999
Caffeic acid	0.095	0.998
p-Coumaric acid	0.047	0.999
Ferrulic acid	0.024	0.999
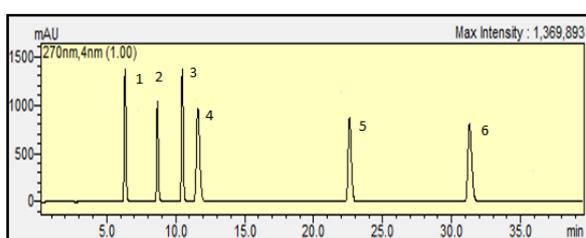
Figure 1. The HPLC chromatogram of standard mixtures: 1. Protocatechuic acid, 2. 4-Hydroxy benzoic acid, 3. Vanillic acid, 4. Caffeic acid, 5. p-Coumaric acid, and 6. Ferrulic acid
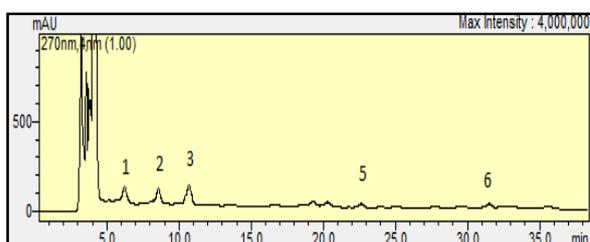
Figure 2. The HPLC chromatogram of sample for ASE 2/1

Among these compounds, vanillic acid (Compound 3) showed the highest content (129.06 mg/1.000 g) in *E. angustifolia* leaves using ASE method (ASE 2/1). Protocatechuic acid (Compound1).

The content of the ASE 2/1 sample was 92.58 mg/1.000 g of methanolic extract, whereas the 4-hydroxy benzoic acid (Compound 2) was (57.88 mg/1.000 g extract). Compounds 5-6 content ranged from 1.76 to 8.69 mg/1.000 g extract. ASE 1/1 sample included 4-hydroxybenzoic acid (95.48 mg/1.000 g) as the main phenolic compound.

Even caffeic acid was not detectable in extracts by Soxhlet and all ASE methods. p-coumaric acid in the cinnamic group was less abundant in all the extracts. Based on the Soxhlet extraction results, protocatechuic acid was found to be a major phenolic acid present in the leaves of *E. angustifolia* with methanolic extract. Hence, the results indicated that the HPLC examination of phenolic acids in the Russian olive leaves was the most abundant benzoic group (**Figure 3**).
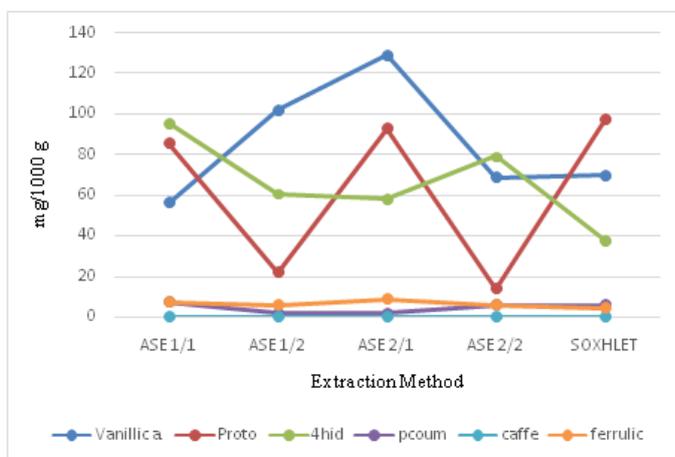
Figure 3. Quantities of phenolic acids in different extracts

α-Glucosidase and α-Amylase Inhibitory Activities

For our scientific evaluation of the claimed effects for *Elaeagnus angustifolia* leaves on diabetic conditions in Turkish folk medicine, methanolic extracts were prepared from the dried leaves by the two methods employed [Soxhlet and ASE] and subjected to the inhibitory effects for antidiabetic activity using α-glucosidase and α-amylase enzymes. The results on the dose-dependent alteration in α-glucosidase and α-amylase inhibitions for extracts are depicted in **Figure 4** and **Figure 5**. Varying concentrations were tested, plant extract which was prepared by different techniques, showed similar α- glucosidase inhibitory activity. The results show that extracts (ASE 1/1, ASE 1/2, ASE 2/1, ASE 2/2 and Soxhlet Extraction) inhibited α-glucosidase in dose-dependently mannerly (10-50 µg/ml) except extract by Soxhlet apparatus.

In this study, we further analysed that vanillic acid was the most abundant phenolic acid of *E. angustifolia* leaves by HPLC from the methanolic extract (ASE 2/1 and ASE 1/2). We
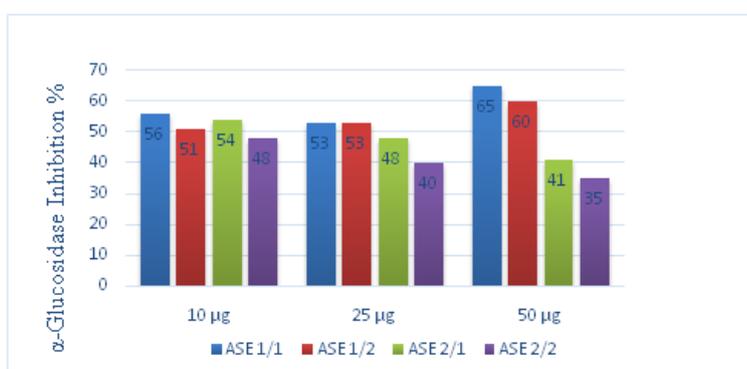

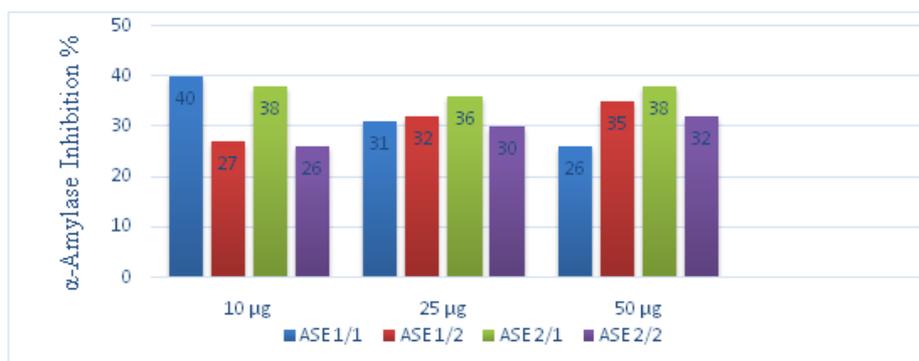**Figure 4.** Soxhlet apparatus results for α-glucosidase inhibition

Figure 5. The results of α-amylase inhibition activity in different extraction methods

found that the most powerful α-glucosidase activity was from ASE 1/1 extract (56 % 10 µg ml^{-1}). Phenolic compounds isolated the from Sea buckthorn (*Hippophae rhamnoides*) and indicated that the butanol fraction included the mostamount of phenolics in UPLC analyses and also the most significant α-glucosidase inhibitory activity [3]. In the present study, it could not be detected even at the higher concentrations of 100 and 250 µg/ml-both of the activities in all extracts and the highest in α-amylase inhibition activity obtained at the concentration of 10 µg/ml in ASE 1/1 sample. This effect could be due to the result of bioactive compounds that are found in *E. angustifolia* leaves.

CONCLUSIONS

In this study, we provide some evidence that α- glucosidase and α-amylase enzyme inhibitör activities using methanolic extracts of *E. angustifolia* leaves. Using HPLC, we elucidated that there is phenolic composition in *E. angustifolia* leaves, which could be helpful in preventing the development of diabetic neuropathy, presumably by blocking the methyl glyoxal-mediated intracellular glycation system.

Vanillic and caffeic acids are found in the ethanolic extract of *E.angustifolia* fruits [24]. The stored commercial oleaster fruits contain seven phenolic acids in their methanolic solution by HPLC [10]. While 4-hydroxybenzoic acid in the benzoic group and caffeic acid in the cinnamic group were the most abundant phenolic acids (45.8 and 32.0 mg/100 g, respectively), benzoic acid in the benzoic group and ferrulic acid in the cinnamic group were less abundant (11.6 and 2.3 mg/1.000 g, respectively) in the HPLC analyses [25].

Hydrolyzed sample of the soft extract of oleaster young branches includes gentisic and caffeic acid by mass spectrum (MS) [12]. Even, the quantities of ferrulic acid and p-coumaric acid were determined 21.10 – 66.22 mg (%) and 144.77- 237.30 mg (%), respectively [26]. The presence of phenolic compounds in theseed crude ethanolic extractfromthe *Hippophae rhamnoides* (Elaeagnaceae) in high performance thin layer chromatography (HPTLC) method [27].

In the peer-reviewed literature, it has been reported that the chloroform fraction of the 75% ethanol extract of the barks of *Cyclocarya paliurus* had the better α-glucosidase inhibitory effects among the other six fractions.

Our findings are consistent with the trend of their benzoic group (especially vanillic and 4-hidroxybenzoic acid) contents and their α-glucosidase and α-amylase inhibitory properties. Thus, the presence of benzoic groups in all phenolic acids in all the extracts might be responsible for the two inhibitory activities of phenolic acid-rich extracts in the leaves of oleaster. Vanillic acid might help in the prevention of the development of diabetic neuropathy by blocking the methylglyoxal-mediated intracellular glycation system [28]. Hence, these previous studies supportive of our working hypothesis. Of course, additional anti-diabetic activity studies and evaluations need to be done in the clinical trialsetting for supplementary treatments of diabetic patients.

In conclusion, our results confirmed the folkloric use of *E. angustifolia* leaves in Turkey. As noted above, some studies inhibitory activities against α-glucosidase and α-amylase of phenolic compounds may be helpful to use these extracts against diabetes [16, 29, 30]. Also, our findings are in agreement with the claims that plant phenolic acids are mild inhibitors of α-amylase and strong inhibitors of α-glucosidase. It has been planned to scan other medicinal or aromatic plants including phenolic compounds and traditionally used as antidiabetic and their mechanisms in our work.

CONFLICTS OF INTEREST STATEMENT

The authors declare that there is no conflict of interest associated with this manuscript. Our study was carried out by our own means and resources.

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