The Chemical Composition and Antidiabetic, Neuroprotective and Cytotoxic Activities of Soft Hulls (Mesocarp) of Pistachio (*Pistacia vera*) Fruits

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Summary: Pistachio (*Pistacia vera* L.) is an important cultivated plant in the Southeastern Anatolia region and its fruits are widely used in the food industry. The previous studies mostly focused on the food minerals and components. However, there is no study on chemical components and biological activity of the soft hulls of the pistachio fruits. In this study, it was focused on the acetone and water extracts of the soft hulls of pistachio fruits. Hence, the acetone extract was subjected to sephadex LH-20 column chromatography and fractioned into five fractions (*Frs. A-E*) according to molecular weight of components. A triterpen compound isolated from the *Fr. B* by crystallation method and its chemical structure was characterized as masticadienonic acid by spectroscopic methods. The fractions contain a mixture of the anacardic acids 13:1, 15:2, 15:1 and 17:1 and their derivatives according to their spectroscopic data and GC-MS analysis. Shikimic acid used in the synthesis of the oseltamivir, antiviral agent was also purified at high yield (14.36%) from the water extract of the soft hulls of pistachio fruits.

In order to explore the antidiabetic properties of the pure compounds, the extracts and the fractions, their inhibition effects were determined on the activities of digestive enzymes (α-glucosidase and α-amylase). All of the extracts, masticadienonic acid and the fractions containing the anacardic acids strongly inhibited the α-glucosidase activity much more than acarbose. However, all applications exhibited much weaker inhibitory properties againts α-amylase, as compaired to the inhibition effect of the acarbose. Furthermore, the treatments of mesocarps of Pistachio fruits acted as much weaker inhibitors against AChE, whereas the acetone extract and its fractions containing different rates of anacardic acids were powerful agents against BChE as strong as neostigmine and galantamine. The cytotoxic activities of the extracts and the compounds against HUVEC, A549 and H1299 cells were also determined using the MTT analysis method and it was determined that the acetone extract and the compounds, masticadienonic and shikimic acids showed cytotoxic effects on the cells.

Keywords: *Pistacia vera*, Soft hulls, Anacardic acids, Shikimic acid, Antidiabetic, Cytotoxic.

Introduction

Diabetes, a common chronic disease in the world is characterized as high blood glucose levels due to both insulin production failure and insulin resistance. Nowadays, it is forecasted that over 300 million people suffer from this disease and about 5 million people have died from the diabetes and diabetes-related diseases [1]. Diabetes is a common disease in Türkiye and it is estimated that there are around 8 million diabetes patients according to the World Health Organization (WHO) reports [1]. In general, there are two types of diabetes, insulin-dependent diabetes (type I) and non-insulin-dependent diabetes (type II) and a relatively small proportion (about 10%) of diabetic patients suffer from type I. On the other hand, approximately 80-90% of the diabetic patients are the non-insulin dependent diabets (type 2) [1, 2]. It has also been reported that the patients suffer from the diabetes have a higher risk of some chronic diseases such as nephropathy, atherosclerosis, and ischemic heart diseases [1, 2]. The digestive enzymes, α-amylase and α-glucosidase are responsible for the increase of post-feeding blood glucose levels [3, 4]. Blood glucose levels can be kept under control with an appropriate diet and exercise program in most of the diabetic patients. However, pharmacological treatment is inevitable in type II diabetic patients and prevention of hyperglycemia inhibiting the digestive enzymes (α-amylase and α-glucosidase) emerges as an important strategy in the treatment of type 2 [3,4].

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Alzheimer's disease (AD) is one of the neurodegenerative disease encountered in elderly people. It is estimated that about 20 million people in the world and about 250 thousand people in Türkiye are suffering from this disease [5, 6]. AD, which constitutes 80% of dementia cases manifests itself with memory loss, dementia and a decrease in cognitive functions due to the death of brain cells over time. Although the exact cause of AD is unknown, it has been determined that autoimmune reactions, genetic factors and accumulation of beta-amyloid plaques in the brain are closely related to the disease. Cholinesterase enzymes catalyze the decomposition reactions of various choline types such as acetylcholine, butyrylcholine and acetylthiocholiniodide. Cholinesterases are a wide distribution in plasma and other body fluids and tissues. Acetylcholinesterase (AChE) is one of the most known cholinesterase enzymes [7]. AChE is found in high concentrations in the brain and erythrocytes, whereas butyrylcholineesterase is found in the serum, pancreas, liver and central nervous system. AChE hydrolyzes acetylcholine to choline and acetic acid, and inhibition of this enzyme results in increasing the acetylcholine level. This is still an effective strategy in the therapy of AD and thus, cholinesterase inhibitors have been used for the cure of this disease from the 1990s [7].

Cancer is one of the leading causes of death and therefore an important public health problem worldwide. It has been reported that about 10 million people died from cancer in 2020 alone [8]. Lung carcinoma is the most common fatal cancer type in the world although its incidence and mortality vary widely among countries. Nowadays, the statistic data pointed out that 11.4% of total cancer cases and 18.0% of cancer deaths are caused by lung cancer [9]. Chemotherapy and radiation therapy are the most common clinical treatment strategies however, many of these chemotherapeutic drugs have significant toxic side effects and drug resistance [10]. Although many natural compounds are used as chemotherapeutic agents [11], there is a need for the develop of new natural chemotherapeutic agents. Therefore, research is ongoing to find more effective natural products that provide fewer negative side effects.

The *Pistacia* (Anacardiaceae) species are distributed in Asia, Europe, Africa and the America. The most widespread species in the flora of Türkiye are *P. vera*, *P. terebinthus*, *P. lentiscus* (mastic tree) and *P. khinjuk*. Due to the widespread use of *P. vera* fruits as food and in the food industry, it is widely grown in the Southeastern Anatolia [12]. Pistachio fruits are known as locally "Antep Fıstığı" and "Şam Fıstığı". Iran, USA and Türkiye are the leading countries in the cultivation of pistachios. Many studies have shown that pistachio fruits and other parts have various pharmacological activities including anti-inflammatory, antiglycemic, blood pressure stabilizing, cholesterol lowering, antiprotozoal, antibacterial, antifungal, antiviral, anticancer, cardioprotective, antilipase and antilipoxygenase [13-19]. Previous reports on the pistachio fruits showed that it contains phenolic cardanols such as 3-(8-pentadekenyl)phenol, 3-(10-pentadekenyl) phenol, 3-pentadecylphenol and 3-(10-heptadekenyl) phenol and anacardic acids 13:1, 13:0 and 17:1 [20-27]. Some reports also demonstarated that the fuits of pistachio are rich in terms of various phenolic antioxidants such as apigenin, caffeic acid, catechin, *p*-coumaric acid, epicatechin, erioditol, erioditol-7-*O*-glycoside, gallic acid, hesperidin, hexagalloyl hexose, *p*-hydroxybenzoic acid, isoramnetin-7-*O*-glycoside, isoramnetin-3-*O*-glycoside, campferol, monogalloyl glycoside, monogalloyl kunic acid, naringenin-7-*O*-neohesperidoside, naringin, penta-*O*-galloyl-β-D-glycoside, protocatechic acid, quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glycoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-rutinoside and syringic acid [16, 18, 21-30]. It has been found that polyphenol-rich extracts of peel and fruits of pistachio significantly reduced lipopolysaccharide-induced inflammation and the production of TNF-α and IL1-β cytokines [31]. In an another study on the pharmacological properties of the pistachio, the protective effect of hydroalcoholic extract of *P. vera* fruits against gentamicin-induced nephrotoxicity in rats was also investigated [32]. Furthermore, it was reported that the methanol extract obtained from the edible fruit part of pistachio significantly reduced atherosclerotic lesions in rabbits, increased HDL cholesterol levels, decreased lipid peroxidation and decreased ALT and AST levels in rabbit blood samples [33]. The ethyl acetate extract of the fruit soft peel have cytotoxic effects against MCF-7, HT-29 and HCT-116 cancer cell lines stimulating apoptosis and inhibiting angiogenesis [19, 34]. Hence, in the current study, we aimed to a) the analyse the chemical constituents of the soft parts of the pistachio fruits by spectroscopic methods b) determine the antidiabetic potentials by testing on the α-glucosidase and α-amylase enzymes activities and c) evaluate the neuroprotective effects by testing on the AChE and BChE enzymes activities and d) test the cytotoxic activities in lung cancer cells by MTT method to explore new pharmacological properties of the soft hulls of pistachio fruits.

Experimental

Chemicals

The solvents used in the experiments were purchased from Sigma-Aldrich, Merck and Tekkim. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium

bromide (MTT) was obtained from Sigma (St Louis, MO, USA). Fetal bovine serum (FBS) and phosphate buffer saline (PBS) were purchased from Life Technologies (Grand Island, NY). Dimethyl sulfoxide (DMSO), and Trypan blue stain solution (0.4%) were purchased from Sigma-Aldrich, (USA). Trypsin/EDTA 0.25% was provided from Invitrogen, (USA) Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco (USA). The cell lines, H1299, A549, HUVEC, were gifted by Prof. Ulukaya from the Department of Medical Biochemistry, Istinye University, Türkiye.

Column chromatography (CC) was performed using silica gel (Merck, 70-230 mesh) and sephadex LH20 (Aldrich/Sigma), whereas thin layer chromatography (TLC) was applied on silica gel 60F-254 (Merck). The spots on TLC belonging to constituents were determined by UV_{365}, UV_{254}, spraying (1% vanillin-H_2SO_4) and heating (105 ºC). The FT-IR (4000-400 cm^{-1}) spectra were documented by an Agilent (Cary 600 Series) instrument. The ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), 1D and 2D-NMR spectra were recorded using a Bruker 400 spectrometer. DMSO-d6 (Merck) and $CDCl_3$ (Merck) were used as solvents, and tetramethylsilane (TMS) (Merck) was used as an internal standard for the NMR assays. The enzyme inhibitions and antioxidant assays were read via a T80+UV PG Instrument.

Plant material and extraction, fractionation and isolation procedures

Soft hulls of the ripened pistachio fruits were acquired in sufficient quantity (September 2018). The dried samples at room condition were ground via a blender (Lab companion, VM-96E) and then the samples (500 g) were macerated with acetone (5 x 2.5L) for 24 h. The extracts were filtered by a filter paper, the filtrates combined and acetone was evaporated using a rotary evaporatory under reduced pressure and temperature (45 ºC) to concentarate the extract. Thus, a viscous liquid extract was obtained (57.0 g, 11.40% yield) [35,36].

The powdered soft hulls (100 g) were extracted with distilled water (800 mL) for 24 hours at room temperature. The extraction process was repeated twice for the same sample. The extract was liyophilizated and thus 25.56 g (25.56%) extract was obtained. To isolate a major compound in the water extract, it was dissolved in the distilled water (100 mL) and 15 grams of silica gel (63-200 µm) was added. The water was removed by a lyophilizer to adsorbe onto silica gel. The extract adsorbed onto silicagel was subjected to the silica gel CC (150 g, 70-30 mesh) and eluted with acetone until lightening. Elution was then continued with methanol and the fractions were checked with TLC [35, 36]. The fraction contained the major compound were collected and the solvent was removed in the rotary evaporator. Thus, a slightly yellow compound was obtained. In order to decolorize the compound, it was redissolved in 100 mL of pure water and 3 g activated carbon was added on it. The mixture was stirred at 50 °C for 2.5 hours and then was filtered over silica gel. A white amorphous solid (14.36 g, 14.36% yield) after the water was lyophilized [35, 36]. The chemical structure of this compound was characterized as shikimic acid by spectroscopic methods.

Based on our previous knowledge that the acetone extract of the soft hulls is rich in the anacardic acids, therefore it (33.70 g) was fractioned over sephadex LH-20 (100 g) column chromatography. The eluents were checked in TLC and acetone extract was fractioned into five sub-fractions (*Fr. A*, 16.54 g), *Fr. B* (4.68 g), *Fr. C* (2.79 g), *Fr. D* (7.40 g) and *Fr. E* (5.34 g). In the control of fraction A with TLC, it was determined that this fraction contains a terpenic compound besides the anacardic acids. Fr. A was dissolved in methanol and kept in the freezer (-4 ºC) for three days and thus, the terpenic compound was precipitated. This process was repeated 5 times and the terpenic substance was separated from other compounds, the anacardic acids. The chemical structure of this terpenic compounds was characterized as masticadienonic acid by spectroscopic methods.

Gas chromatography-mass spectrometry (GC-MS)

The chemical compositions of the fractions containing the anacardic acids obtained from the soft hulls with sephadex CC were analyzed using the Agilent 7890B device attached to a DB-WAX capillary column (30 m x 0.25 mm i.d., 0.50 µm). An electron ionization (E.I) system (70 eV) was used for mass analysis. The carrier gas in the column was helium (2 mL/min). Injection and MS transfer temperatures were 200 ºC and 250 ºC, respectively. Column temperature was rised from 50 °C to 250 °C with 10ºC/min, and was held isothermal at 250 °C for 20 min. An 1 µL of the fractions diluted in chloroform (1/10, mass/volume) was automatically injected at a split ratio of 20:1. The relative percentage of components was determined by an automatic integration of the device. The qualitative analysis of each compound was performed by comparing those of the NIST libraries of the mass spectra in the DB-WAX capillary column.

Enzyme inhibition assays

The details of the enzyme inhibitory assays for α-glucosidase, α-amylase, AChE and BChE enzymes have been reported in our previous reports [35,36].

Cell lines, cell cultures and cell viability assays

Human umbilical vein endothelial cells (HUVECs; CRL-1730), H1299 (CRL-5803) and A549 (CCL-185) non-small cell lung cancer cell lines were cultivated in DMEM supplemented with 10% FBS, antibiotic-antimycotic solution, which contains penicillin (100 IU/mL), streptomycin (100 µg/mL). The cells were maintained at 37 °C, in a 5% CO_2 atmosphere with 95% humidity. The medium was changed and cells were detached with trypsin/EDTA 0.25% and sub-cultured as they reached 70% confluence [37].

The cytotoxic effects of the extracts, the fractions and the compounds on A549, H1299 and HUVEC cell lines were based on the MTT method. Briefly, the cells at 5×10^3 in 100 µL medium were added into each well of a 96-well plate and cultured in triplicate in the presence or absence of various concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) of the soft hulls treatments. After incubated 48 and 72 hours, the MTT solution (40 µL, 5 mg/mL in PBS) of was added to each well and the cells were further incubated for 4 h. MTT solutions were replaced with 80 µL of DMSO and incubated for 20 min to dissolve formazan crystals and then, the optical density of each well was measured at the wavelength of 570 nm using a microplate reader (Multiskan FC, Thermo). From the average absorbance values for the treatments and controls, the percent cells viability were established by the following equation:

Cell viability (%) = [(optical density of treated cells-optical density of blank)/(optical density of control cells-optical density of blank)] x 100.

Half-maximal inhibitory concentrations (IC_{50}), the concentrations of the tested samples required to inhibit 50% cell proliferation, were calculated from the mean values of data from wells [38].

Statistical analyses

The data obtained from the bioassays were subjected to the one-way analysis of variance (ANOVA), via the SPSS 17.0 software package to determine whether there is a statistically difference. LSD and Duncan tests were performed to determine the differences between means for the enzyme inhibition assays and the values of $p<0.05$ were considered significantly different. IC_{50} and IC_{90} values of each application were calculated using Microsoft Excel program for the enzyme inhibition assays. Statistical analyzes for the cytotoxic activity findings were performed using One sample t test in SPSS program and IC_{50} values were calculated with Microsoft Excel (Windows 2010).

Results and Discussions

The chemial composition of the extracts and the fractions

In the present study, 5 fractions (*Frs. A-E*) containing anacardic acids were obtained from the acetone extract of the soft hulls of the pistachio fruits of by sephadex LH-20 column chromatography. In the TLC analysis of the *Fr. A*, it was determined that it contains a terpenic substance besides phenolic compounds. The terpenic compound was isolated from the other phenolics by cold precipitation (-4 ºC) and its chemical structure was characterized as masticadienonic acid by IR, ^{1}H-NMR, ^{13}C-NMR, 1D- and 2D-NMR spectroscopic methods. Masticadienonic acid is the characteristic triterpene found in the resin and other parts of the various *Pistacia* species [17, 23, 35, 39, 40]. The current results suggest that this compound is also present in the soft hulls of *P. vera* fruits. According to ^{1}H-NMR, ^{13}C-NMR, 1D- and 2D-NMR spectral data of the *Frs. A-E*, it was observed that these fractions contain anacardic acid derivatives. On the other hand, there are some differences about the NMR spectral data of the *Fr. A* in comparison to those of *Frs. B-E*. These spectral data indicate that anacardic acids in the *Fr. A* contains additional polar groups such as polar -OH groups and an ester group. In the ^{1}H-NMR spectrum, the signals of the H atoms adjacent to the –OH group were observed at δ=4.21 ppm (*d*, *J*=4.86 Hz), δ=3.47 ppm (*t*, *J*=5.24 Hz and 5.40 Hz) and δ=3.35 ppm (*t*, *J*=6.64 Hz and 6.28 Hz). In the ^{13}C-NMR spectrum of *Fr. A*, the additional signals observed at δ=165.4 ppm, δ=134.2 ppm, δ=72.5 ppm, δ=70.5 ppm, δ=67.5 ppm and δ=60.7 ppm also provide evidence that the anacardic acids in the *Fr. A* contain additional groups when compared to the ^{13}C-NMR spectra of other fractions Four positive signals observed at δ=60-75 ppm in the APT ^{13}C-NMR spectrum of the Fr. indicate that these groups are –CH_2-O- groups. These spectral data indicate that the phenolic compounds in the *Fr. A* are derived from –COOH or -OH (Fig. 1).

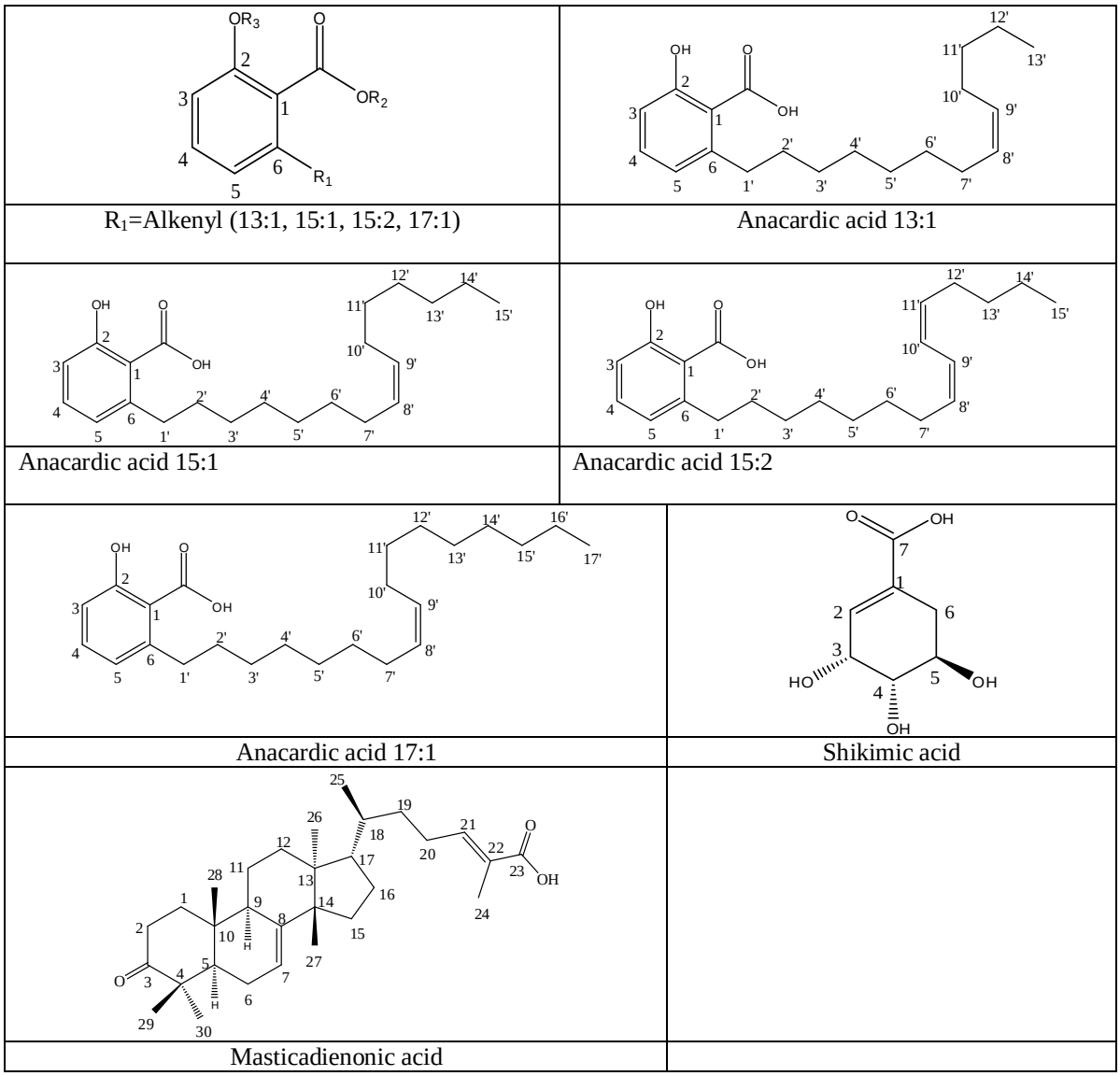


Fig. 1: The chemical structures of shikimic acid isolated from the water extract and masticadienonic acid, anacardic acids and their derivatives in the acetone extract of the soft hulls of pistachio fruits.

The chemical constituents of the acetone extract of the soft hulls and the *Frs. A-E* were analysed by GC-MS and the results are summarized in Table-1. GC-MS analyses showed that the acetone extract and its fractions contain the different relative amounts of the anacardic acids 13:1, 15:1, 15:2 and 17:1 and their derivatives (Fig. 1). Anacardic acid 17:1 is the major component of the acetone extract, Frs. A and E. These results suggested that *Fr. A* contains mainly the polar derivatives of the anacardic acid 17:1. However, *Frs. B* and *C* consist of anacardic acids 15:1 and 15:2

Shikimic acid was purified as a amorphous white solid from the water extract of the soft hulls with a high yield (14.86%) and its chemical structure was characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, 1D- and 2D-MR spectroscopy. It is an intermediate metabolite in the synthesis of phenolic compounds in plants and microorganisms [41-43]. The plants contain varying amounts of shikimic acid, however the best source for shikimic acid isolation is star anise (*Illicium* sp.) [41-45]. Shikimic acid is commonly used in the synthesis of the antiviral drug agent, oseltamivir [43-45]. In this study, this compound was isolated from the water extract of the mesocarps of pistachio fruits with higher yields than *Illicium* sp.

Table-1: The chemical compositions of the acetone extract and its fractions (*Frs. A-E*) of the soft hulls.

Rt	Components	Whole extract	*Fr. A* (%)	*Fr. B* (%)	*Fr. C* (%)	*Fr. D* (%)	*Fr. E* (%)
22.792	**13:1**	**11.35**	**27.61**	**-**	**-**	**28.04**	**7.22**
25.863	**15:2**	**31.62**	**5.33**	**44.75**	**68.59**	**37.81**	**14.82**
27.087	**15:1**	**16.29**	**9.30**	**53.70**	**25.43**	**34.14**	**12.04**
37.354	**17:1**	**40.74**	**57.77**	**1.55**	**-**	**-**	**65.91**

Rt: retention time

Antidiabetic effects of the extracts, the fractions and metabolites of the soft hulls of pistachio fruits

Since the existence of the humanity, mankind have searched the possibilities of being treated with natural resources by using their intelligence, research and learning skills. Plants hold the most important place among these natural resources and over the years, the knowledge of people about the therapeutic properties of plants against different diseases has increased [46-48]. Recently, some aromatic and medicinal plants have been cultivated for their nutritional, flavor and aroma, appetizing and therapeutic effects [47, 48]. Determination of enzyme activity is a very important process in the definition of various diseases [49, 50]. Since many drug active ingredients show their effects through enzyme inhibition, inhibitors are very important tools in elucidating both enzyme action mechanisms and metabolic pathways [49, 51]. Diabetes mellitus, a common chronic disease, is characterized by high blood sugar due to both insulin insufficiency and insulin resistance. Furthermore, it is increasing day by day in the world due to the change in diet. Type 2 diabetes (non-insulin dependent) covering about 80-90% of diabetic patients is much more common type than type 1 diabetes (insulin-dependent) [1,2]. Today, the most preferred treatment for type 2 diabetes is to reduce hyperglycemia via inhibition of the digestive enzymes, α-glycosidase and α-amylase after feeding [3,4].

The extracts, the fractions and pure compounds (masticadienonic acid and shikimic acid) were tested in vitro on the α-glucosidase and α-amylase enzymes activities to evaluate the antidiabetic potentials of the soft hulls. The antidiabetic properties of all tested reagents were compared with that of the antidiabetic agent, acarbose [1, 35, 36] (Tables 2-4). IC_{50} and IC_{90} values were also determined to compare the antidiabetic activity of the pistachio applications and the acarbose against α-glucosidase enzyme and low IC_{50} or IC_{90} values indicate a high inhibition effect (Table-5). As shown in these tables, all pistachio applications are more effective against the α-glucosidase enzyme than the α-amylase enzyme and the low concentrations of the all applications exhibited significant inhibitory effects on the α-glucosidase activity in dependent concentrations. Moreover, all of the applications except for shikimic acid were found to have a stronger inhibitory effect than acarbose (Tables 2-4). The extracts, masticadienonic acid and the fractions exhibited very strong inhibitory effects at lower concentrations (0.01-5 mg/mL), whereas the acarbose inhibited the α-glucosidase enzyme activity in the range of 5-80 mg/mL with 37.37-89.65%. In particular, the acetone extract and the fractions rich in anacardic acids and their derivatives exhibited a very strong inhibitory effect against α-glycosidase enzyme at very low concentrations (IC_{50}=0.01-1.17 mg/mL). These results show that the anacardic acids and their derivatives are potent inhibitory agents against α-glucosidase [23]. The water extract and shikimic acid isolated from the water extract was found to be weaker inhibitors against α-glucosidase enzyme with the higher IC_{50} values (2.12 mg/mL and 85.25 mg/mL, respectively) as compared to the acetone extract and its fractions (Table-5).

In contrast to the results for α-glucosidase enzyme inhibitory assays, all of the pistachio applications were found to have a weak inhibitory effect (IC_{50}=7.25-131.28 mg/mL) than acarbose (IC_{50}=0.11 mg/mL) on the α-amylase enzyme (Tables 1-5). In accordance with the literature [35,52], in the present study, acarbose was found to be more potent inhibitor against α-amylase as compared to α-glycosidase. Among the pistachio applications, the most active applications against α-amylase activity are the acetone extract and *Fr. 5* with IC_{50}=7.25 and 4.77 mg/mL, respectively. The stronger inhibitory property of the *Fr.* 5 can be attributed to the high content (65.91%) of the anacardic acid 17:1 (Table 1) as compared to the oher fractions of the acetone extract. However, the water extract and shikimic acid isolated from the water extract acted as much weaker inhibitors (IC_{50}=122.47 and 131.28 mg/mL, respectively) when compared to the inhibitory effects of acetone extract and its fractions rich in anacardic acids. Our results indicated that the antidiabetic effect of the soft hulls of pistachio fruits is closely related to its anacardic acid components.

The common side effects of commercial antidiabetic agents such as acarbose, vaglibose, and miglitol are the diarrhea, abdominal distention, and flatulence due to excessive inhibition of pancreatic amylase [53, 54]. Therefore, high inhibition of α-

glucosidase and low inhibition of α-amylase in the treatment of type 2 diabetes is a preferable approach to reduce these side effects of the antidiabetic agents [53-56]. As can be seen from Table 5, unlike acarbose, all pistachio applications except for shikimic acid application displayed a high α-glucosidase inhibition and low α-amylase inhibition.

Table-2: The antidiabetic properties of the acarbose, acetone extract and masticadienonic acid isolated from the soft hulls of pistachio fruits.

	α-Glucosidase			α-Amylase		
Treatments	**Conc. (mg/mL)**	**Abs±SE**	**% Inh.**	**Conc. (mg/mL)**	**Abs±SE**	**% Inh.**
Enzyme + substrate	**-**	**0.396 ±0.006fg**	**-**	**-**	**0.462±0.004e**	**-**
Enzyme + substrate + acarbose	**1.0**	**0.353±0.009f**	**10.9**	**0.025**	**0.382±0.002d**	**17.3***
	2.5	**0.340±0.008f**	**14.1**	**0.05**	**0.327±0.003c**	**29.2***
	5.0	**0.288±0.005e**	**27.3***	**0.1**	**0.225±0.001c**	**51.3***
	10	**0.205±0.003d**	**48.2***	**0.2**	**0.107±0.002b**	**74.7***
	20	**0.104±0.010c**	**73.7***	**0.4**	**0.000±0.000a**	**100.0***
	40	**0.000±0.0030a**	**100.0***			
Enzyme + substrate	**-**	**0.419±0.005d**	**-**	**-**	**0.394±0.013f**	**-**
Enzyme + substrate + extract	**0.005**	**0.332±0.002c**	**20.8***	**1**	**0.282±0.008e**	**28.4***
	0.01	**0.232±0.005b**	**44.6***	**2.5**	**0.251±0.004d**	**36.3***
	0.025	**0.030±0.001a**	**92.8***	**5**	**0.224±0.004c**	**43.2***
	0.05	**0.000±0.000a**	**100.0***	**7.5**	**0.204±0.007bc**	**48.2***
				10	**0.174±0.006b**	**55.8***
				20	**0.010±0.000a**	**97.5**
Enzyme + substrate	**-**	**0.522±0.003**	**-**	**-**	**0.359±0.002f**	**-**
Enzyme + substrate + masticadienonic acid	**0.1**	**0.500±0.006**	**4.2**	**10**	**0.313±0.001e**	**12.8***
	0.25	**0.478±0.005**	**8.4**	**20**	**0.265±0.004d**	**26.2***
	0.5	**0.392±0.008**	**24.9***	**40**	**0.183±0.009c**	**49.0***
	1	**0.244±0.004**	**53.3***	**60**	**0.072±0.002**	**79.9***
	2.5	**0.000±0.003**	**100.0***	**80**	**0.009±0.001**	**97.5***

* Statistically different from control (enzyme + substrate) ($p<0.05$). Different letters in the lines are statistically different according to Duncan test.

Table-3: The antidiabetic properties of the fractions obtained from acetone extract of the soft hulls of pistachio fruits.

	α-Glucosidase			α-Amylase		
Treatments	**Conc. (mg/mL)**	**Abs±SE**	**% Inh.**	**Conc. (mg/mL)**	**Abs±SE**	**% Inh.**
Enzyme + substrate	**-**	**0.542±0.003d**	**-**	**-**	**0.261±0.008d**	**-**
Enzyme + substrate + *Fr. A*	**0.01**	**0.354±0.009c**	**34.7***	**10**	**0.222±0.005d**	**14.9***
	0.02	**0.153±0.009b**	**71.8***	**20**	**0.180±0.007c**	**31.1***
	0.03	**0.014±0.002a**	**97.4***	**40**	**0.152±0.003b**	**41.6***
	0.04	**0.000±0.000a**	**100.0***	**80**	**0.057±0.020a**	**78.1***
Enzyme+ substrate	**-**	**0.489 ±0.003c**	**-**	**-**	**0.396 ±0.008d**	**-**
Enzyme + substrate + *Fr. B*	**0.01**	**0.473±0,019c**	**3.3**	**10**	**0.373±0.015d**	**5.8**
	0.02	**0.319±0,004b**	**34.8***	**20**	**0.305±0.005c**	**22.9***
	0.04	**0.070±0,001a**	**85.7***	**40**	**0.271±0011b**	**31.6***
	0.06	**0.000±0,000a**	**100.0***	**80**	**0.126±0,004a**	**68.2***
Enzyme + substrat	**-**	**0.461±0.007de**	**-**	**-**	**0.386±0.006ef**	**-**
Enzyme + substrate + *Fr. C*	**0.01**	**0.280±0.006d**	**39.3***	**5**	**0.334±0.006e**	**13.5**
	0.015	**0.148±0.009c**	**67.9***	**10**	**0.308±0.004d**	**20.2***
	0.020	**0.070±0.003b**	**84.8***	**20**	**0.271±0.007c**	**29.8***
	0.025	**0.016±0.005ab**	**96.5***	**40**	**0.231±0.004b**	**40.2***
	0.05	**0.000±0.000a**	**100.0***	**80**	**0.109±0.004a**	**71.8***
Enzyme + substrate	**-**	**0.549±0.010e**	**-**	**-**	**0.522±0.014e**	**-**
Enzme + substrate + *Fr. D*	**0.01**	**0.540±0.004e**	**1.6**	**10**	**0.333±0.016d**	**36.2***
	0.02	**0.449±0.005d**	**18.2***	**20**	**0.259±0.015c**	**50.4***
	0.03	**0.276±0.014c**	**49.7***	**40**	**0.188±0.009b**	**64.0***
	0.04	**0.087±0.007b**	**84.2***	**80**	**0.012±0.004a**	**97.7***
	0.05	**0.00±0.000a**	**100.0***			
Enzyme + substrate	**-**	**0.554±0.007e**	**-**	**-**	**0.355±0.009h**	**-**
Enzyme + substrate + *Fr. E*	**0.01**	**0.528±0.004e**	**4.7**	**1**	**0.237±0.008g**	**33.2***
	0.015	**0.382±0.007d**	**31.0***	**2.5**	**0.193±0.007f**	**45.6***
	0.02	**0.122±0.004c**	**78.0***	**5**	**0.159±0.003e**	**55.2***
	0.025	**0.018±0.003b**	**96.8***	**10**	**0.116±0.003d**	**67.3***
				20	**0.032±0.005c**	**91.0***
				40	**0.000±0.000a**	**100.0***

* Statistically different from control (enzyme + substrate) ($p<0.05$). Different letters in the lines are statistically different according to Duncan test.

Table-4: The antidiabetic properties of the water extract and shikimic acid of the soft hulls of pistachio fuits.

Treatments	α-Glucosidase			α-Amylase		
	Conc. (mg/mL)	Abs±SE	% Inh.	Conc. (mg/mL)	Abs±SE	% Inh.
Enzyme+ substrate	-	0.268±0.005f		-	0.389±0.008e	-
Enzyme + substrate +extract	0.1	0.258±0.011f	3.7	10	0.380±0,010de	2.3
	0.25	0.234±0.007ef	12.7	20	0.358±0.005d	8.0
	0.5	0.225±0.011e	16.1*	40	0.323±0.009c	17.0
	0.75	0.199±0.008d	25.8*	80	0.235±0.007b	39.6*
	1	0.165±0.005c	38.4*	160	0.148±0.007a	62.40*
	2.5	0.097±0.007b	63.8*			
	5	0.005±0.001a	98.1*			
Enzyme + substrate	-	0.333±0.015	-	-	0.492±0.010e	-
Enzyme + substrate + shikimic acid	10	0.323±0.004	3.0	10	0.466±0.009e	5.3
	20	0.312±0.008	6.3	20	0.441±0.005d	10.4
	40	0.251±0.002	24.6*	40	0.383±0.008c	22.2*
	80	0.168±0.002	49.6*	80	0.314±0.006b	36.2*
	160	0.018±0.007	94.6*	160	0.209±0.005a	55.5*

* Statistically different from control (enzyme + substrate) ($p<0.05$). Different letters in the lines are statistically different according to Duncan test.

Table-5: IC_{50} ve IC_{90} values of the treatments on the α-glucosidase and α-amylase enzymes.

Treatments	α-Glucosidase			α-Amylase		
	IC_{50} (mg/mL)	IC_{90} (mg/mL)	R^2	IC_{50} (mg/mL)	IC_{90} (mg/mL)	R^2
Acarbose	12.29	24.02	0.9822	0.11	0.23	0.9770
Acetone extract	0.01	0.02	0.9923	7.25	18.53	0.9832
Fr. A	0.01	0.03	0.9889	47.48	94.05	0.9840
Fr. B	0.03	0.04	0.9970	58.76	106.33	0.9772
Fr. C	0.01	0.02	0.9603	50.96	104.54	0.9921
Fr. D	0.03	0.05	0.9837	23.24	70.47	0.9919
Fr. E	0.02	0.03	0.9751	4.77	18.87	0.9574
Masticadienonic acid	1.17	2.16	0.9766	39.51	71.83	0.9939
Water extract	2.12	4.22	0.9657	122.47	222.35	0.9751
Shikimic acid	85.25	149.94	0.9950	131.28	247.83	0.9754

Neuroprotective effects of the extracts, the fractions and metabolites of the mesocarps of pistachio fruits

It is estimated that nearly 20 million people in the world have Alzheimer's disease today. Alzheimer's disease gives itself symptoms with some behavioral disorders such as memory, speech, and recognizing people as a result of the gradual loss of cells in some parts of the brain. It was determined that the amount of acetylcholine released from neuromuscular junctions and cholinergic synapses of the central nervous system reduced in Alzheimer's patients, and therefore AChE enzyme inhibitors began to be used in the treatment in the 1970s [57,58]. Besides AChE, BChE enzyme level was found to be higher in the brains of individuals with Alzheimer's disease than in a healthy brain [59]. Therefore, the inhibition of both AChE and BChE enzymes is one of the frequently preferred approaches in the treatment of Alzheimer's disease.

In the present study, the inhibitory effects of the all of the pistachio applications were determine on AChE and BChE enzymes activities to find new natural neuroprotective agents and were compared with those of commercial cholinesterase inhibitors, neostigmine and galantamine (Tables 6-8) [35, 36, 60-62]. IC_{50} and IC_{90} values were computed to compare the inhibitory properties of pure pistachio metabolites, masticadienonic acid and shikimic acid, the extracts and the fractions containing the different amounts of the anacardic acids against AChE and BChE enzymes (Table-9). As shown in the Tables 6-9, *Pistacia* applications exhibited an inhibition effect on the AChE enzyme at very high concentrations (10-640 mg/mL) with the high IC_{50}=68.72-1271.75 mg/mL values as compared with the neuroprotective agents, neostigmine and galantamine (IC_{50}=0.64 and 4.78 mg/mL, respectively). These results postulated that all of the pistachio applications are ineffective or very weak inhibitors against the AChE enzyme. On the other hand, among the pistachio applications, the most effective application with an IC_{50} of 68.72 mg/mL is masticadienonic acid isolated from the acetone extract of the soft hulls of pistachio fruits and shikimic acid is the weakest inhibitor with IC_{50}=1271.75 mg/mL isolated from the water extract (Table-9). Nevertheles, in contrast to the results for AChE enzyme, the acetone extract and its fractions,

and masticadienonic acid exhibited stronger inhibitory effects at low concentrations (0.1-10 mg/mL) on BChE enzyme activity (Tables 6-9). These results indicate that BChE enzyme is more sensitive to the anacardic acids and masticadienonic acid in the acetone extract of the soft hulls as compared to the AChE enzyme. IC_{50} for neostigmine and galantamine were calculated as 0.03 and 0.31 mg/mL, respectively, whereas IC_{50} values for acetone extract and its fractions were determined as 0.09-0.70 mg/mL against BChE enzyme. The present results also showed that masticadienonic acid acted as a weaker inhibitor on the BChE enzyme with an IC_{50}=3.71 mg/mL than the anacardic acids. These results also provide an evidence that the biological activity of the extract and the pure compounds may differ, although the acetone extract and its fractions containing different amounts of anacardic acids and their derivatives exhibit similar inhibitory effects according to their IC_{50} values (Table-9). For instance, the acetone extract strongly inhibited the BChE enzyme with an IC_{50}=0.10 mg/mL, masticadienonic acid isolated from this extract acted as a weaker inhibitor (IC_{50}=3.71 mg/mL). Likewise, as can be seen from Tables 8 and 9, shikimic acid (IC_{50}=112.40 mg/mL) isolated from the water extract was found to be a weaker inhibitor than water extract (IC_{50}=38.93 mg/mL). These results show that synergistic and/or antagonistic interactions among the major and minor consituents in herbal extracts should not be ignored.

In the literature, there have been some reports on the inhibition effects of some *Pistacia* species and their pure metabolites of the *Pistacia* species against cholinesterase enzymes [28, 35, 63-65]. In accardonce with our results, it has been reported the methanol extract of the mesocarps of *P. vera* fruits acted as a remarkable inhibitor against the activity of the AChE enzyme [28]. The results reported by Kilic et al. (2016) were in full agreement with our study, but only extracts were studied in the aforementioned study [28]. In the current study, the chemical compositions of the acetone and water extracts of the soft hulls were also characterized by GC-MS and NMR spectroscopic methods, besides their neuroprotective properties. Likewise, Jazayere et al. (2014) reported the weak inhibitor effect of the methanol-water (1:1) extract of *P. vera* leaves [63]. Ammari et al. (2018) indicated that *P. lentiscus* oil decreased the level of cholinesterase enzymes in the liver [64]. Furthermore, It has been evaluated that ethyl acetate extract of *P. integerrima* reduced the AChE and BChE enzymes activities by 80.80% and 82.56%, respectively.

Table-6: Neuroprotective effects of the commercial agents (neostigmine and galantamine), acetone extract and masticadienonic acid isolated from the soft hulls of pistachio fruits.

	AChE			BChE		
Treatments	**Conc. (mg/mL)**	**Abs±SE**	**% Inh.**	**Conc. (mg/mL)**	**Abs±SE**	**% Inh.**
Enzyme + substrate	**-**	**0.706±0.008e**	**-**	**-**	**0.452±0.010e**	**-**
Enzyme + substrate + neostigmine	**0.25**	**0.430±0.007d**	**39.1***	**0.001**	**0.334±0.008d**	**26.1***
	0.5	**0.361±0.009c**	**48.9***	**0.002**	**0.241±0.008c**	**46.7***
	1	**0.288±0.004b**	**59.2***	**0.004**	**0.143±0.010b**	**68.4***
	2	**0.142±0.008a**	**79.9***	**0.008**	**0.008±0.000a**	**98.2***
				0.016	**0.000±0.000a**	**100.0***
Enzyme + substrate	**-**	**0.703±0.001e**	**-**	**-**	**0.516±0.004f**	**-**
Enzyme + substrate + galantamine	**1.25**	**0.450±0.005e**	**36.0***	**0.1**	**0.346±0.001e**	**33.0***
	2.5	**0.416±0.002d**	**40.8***	**0.25**	**0.258±0.003d**	**50.0***
	5	**0.333±0.004c**	**52.6***	**0.5**	**0.192±0.004c**	**62.8***
	10	**0.217±0.006b**	**69.1***	**0.75**	**0.118±0.007b**	**77.1***
				1	**0.036±0.007a**	**93.0***
Enzyme + substrate	**-**	**0.854±0.008e**	**-**	**-**	**0.783±0.010e**	**-**
Enzyme + substrate + extract	**40**	**0.664±0.007d**	**22.3***	**0.1**	**0.418±0.008d**	**46.6***
	80	**0.560±0.009c**	**34.4***	**0.15**	**0.237±0.008c**	**69.7***
	160	**0.483±0.004b**	**43.6***	**0.2**	**0.096±0.010b**	**87.7***
	320	**0.255±0.008a**	**70.1***	**0.25**	**0.000±0.000a**	**100.0***
Enzyme + substrate	**-**	**0.401±0.011d**	**-**	**-**	**0.598±0.006de**	**-**
Enzyme + substrate + masticadienonic acid	**10**	**0.387±0.004d**	**3.5**	**1**	**0.548±0.005d**	**8.4**
	20	**0.371±0.006d**	**7.5**	**2.5**	**0.408±0.002c**	**31.8***
	40	**0.288±0.004c**	**28.2***	**5**	**0.134±0.04b**	**77.6***
	60	**0.244±0.005b**	**39.2***	**7.5**	**0.008±0.004a**	**98.7***
	80	**0.155±0.005a**	**61.4***	**10**	**0.000±0.000a**	**100.0***

* Statistically different from control (enzyme + substrate) ($p<0.05$). Different letters in the lines are statistically different according to Duncan test.

Table-7: Neuroprotective effects of the fractions obtained from acetone extract of the soft hulls of pistachio fruits.

Treatments	AChE			BChE		
	Conc. (mg/mL)	Abs±SE	% Inh.	Conc. (mg/mL)	Abs±SE	% Inh.
Enzyme + substrate	-	0.609±0.001e	-	-	0.541±0.009e	-
Enzyme + substrate + *Fr. A*	40	0.553±0.005e	9.2	0.1	0.521±0.001e	3.7
	80	0.497±0.002d	18.4*	0.25	0.453±0.003d	16.3*
	160	0.463±0.004c	24.0*	0.5	0.316±0.004c	41.6*
	320	0.328±0.006b	46.1*	0.75	0.256±0.007b	52.7*
	640	0.192±0.004a	68.5*	1	0.159±0.006a	70.6*
Enzyme + substrate	-	0.608±0.005e	-	-	0.510±0.010e	-
Enzyvme + substrate + *Fr. B*	40	0.606±0.002e	0.3	0.1	0.458±0.004e	10.2
	80	0.579±0.009d	4.8	0.25	0.401±0.010d	21.4*
	160	0.480±0.007c	21.1*	0.5	0.268±0.012c	47.5*
	320	0.348±0.006b	42.8*	0.75	0.175±0.005b	65.7*
	640	0.183±0.007a	69.9*	1	0.116±0.008a	77.3*
Enzyme+ substrate	-	0.607±0.010e	-	-	0.636±0.008 f	-
Enzyme+ substrate + *Fr. C*	40	0.606±0.005e	0.2	0.025	0.617±0.011f	3.0
	80	0.580±0.005d	4.8	0.05	0.551±0.003e	13.4*
	160	0.508±0.009c	16.3*	0.075	0.471±0.006d	25.9*
	320	0.393±0.002b	35.3*	0.1	0.299±0.005c	53.0*
	640	0.216±0.005a	64.4*	0.25	0.007±0.004b	98.9*
Enzyme+ substrate	-	0.592±0.008f	-	-	0.530±0.006 e	-
Enzyme+ substrate + *Fr. D*	40	0.553±0.004e	6.6	0.05	0.329±0.006d	37.9*
	80	0.496±0.006d	16.1*	0.1	0.233±0.004c	56.0*
	160	0.462±0.003c	22.0*	0.25	0.109±0.009b	79.4*
	320	0.279±0.003b	52.9*	0.5	0.000±0.000a	95.7*
	640	0.088±0.004a	85.1*			
Enzyme + substrate	-	0.607±0.003f	-	-	0.545±0.010 f	-
Enzyme + substrate + *Fr. E*	40	0.558±0.006e	8.1	0.05	0.420±0.005 e	22.9*
	80	0.497±0.008d	18.1*	0.1	0.304±0.008 d	44.2*
	160	0.418±0.002c	31.1*	0.15	0.146±0.005 c	73.2*
	320	0.227±0.007b	62.6*	0.2	0.069±0.004 b	87.3*
	640	0.059±0.003a	90.3*	0.25	0.000±0.000 a	100.0*

* Statistically different from control (enzyme + substrate) ($p<0.05$). Different letters in the lines are statistically different according to Duncan test.

Table-8: Neuroprotective effects of the water extract and shikimic acid.

Treatments	AChE			BChE		
	Conc. (mg/mL)	Abs±SE	% Inh.	Conc. (mg/mL)	Abs±SE	% Inh.
Enzyme+ substrate	-	0.771±0.006e	-	-	0.387±0.008g	-
Enzyme + substrate+ extract	40	0.756±0.002d	2.0	10	0.310±0.005e	19.9*
	80	0.702±0.006c	9.0	15	0.271±0.007d	30.0*
	160	0.587±0.007b	24.4*	20	0.244±0.007c	37.0*
	320	0.266±0.007a	65.5*	40	0.176±0.002b	54.5*
				80	0.065±0.003a	83.2*
Enzyme + substrate	-	0.811±0.010e	-	-	0.513±0.002d	-
Enzyme + substrate + shikimic acid	40	0.793±0.006e	2.2	10	0.482±0.004d	2.6
	80	0.779±0.006d	4.0	20	0.474±0.005c	7.6
	160	0.744±0.006c	8.3	40	0.438±0.005b	14.6*
	320	0.709±0.003b	12.6*	80	0.331±0.003a	35.5*
	640	0.602±0.005a	25.8*			

* Statistically different from control (enzyme + substrate) ($p<0.05$). Different letters in the lines are statistically different according to Duncan test.

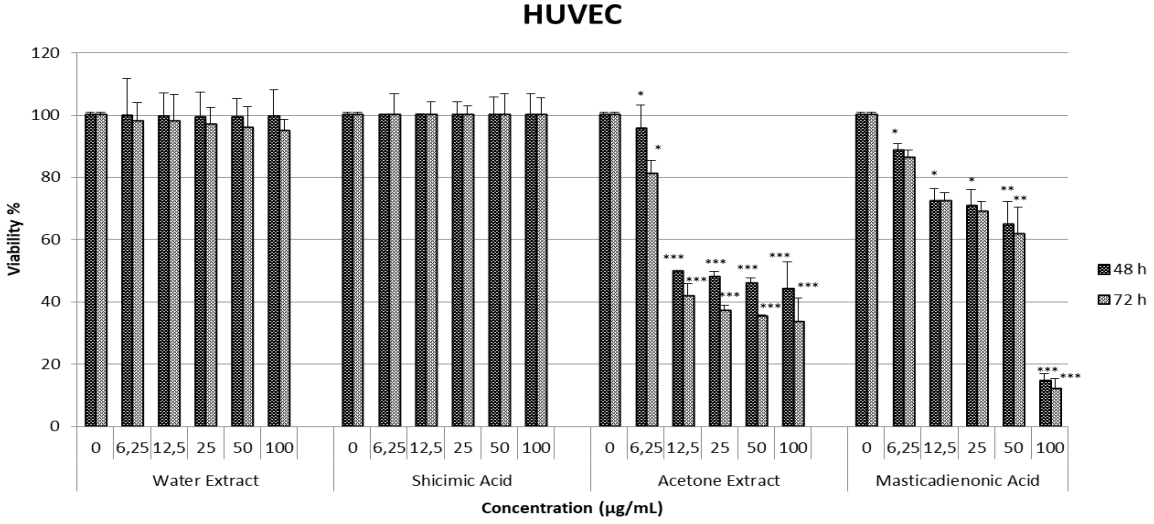
Table-9: IC_{50} ve IC_{90} values of the treatments on the AChE and BChE enzymes.

Treatments	AChE			BChE		
	IC_{50} (mg/mL)	IC_{90} (mg/mL)	R^2	IC_{50} (mg/mL)	IC_{90} (mg/mL)	R^2
Neostigmine	0.64	2.42	0.9887	0.03	0.06	0.9614
Galantamine	4.78	15.31	0.9934	0.31	0.94	0.9863
Acetone extract	195.40	440.95	0.9868	0.10	0.21	0.9820
Fr. A	421.23	834.45	0.9740	0.70	1.24	0.9837
Fr. B	419.10	762.15	0.9725	0.59	1.11	0.9818
Fr. C	489.09	861.88	0.9917	0.13	0.22	0.9572
Fr. D	355.53	667.55	0.9791	0.09	0.30	0.9576
Fr. E	306.34	599.81	0.9618	0.11	0.21	0.9748
Masticadienonic acid	68.72	117.22	0.9867	3.71	6.51	0.9760
Water extract	258.23	432.98	0.9936	38.93	85.56	0.9775
Shikimic acid	1271.75	2310.71	0.9945	112.40	198.18	0.9942

Cytotoxic activities the extracts and the metabolites of the soft hulls of pistachio fruits

The soft hulls of pistachio fruits have caught the attention of researchers in recent years due to its various pharmacological activities including antioxidant [21, 29, 66-68], antimicrobial and antimutagenic [69]. Furthermore, it has been reported pistachio extract induces apoptosis through multiple signaling pathways by causing oxidative stress on colon [70], breast [71] and prostate [72] cancer cell lines. Hence, in this study, cytotoxic effects of the water and acetone extracts and the pure compounds, shikimic and masticadienonic acids against HUVEC, A549 and H1299 cell lines were performed on each cell line after 48h and 72h exposure to increasing concentrations (0, 6.25, 12.5, 25, 50, 100 µg/mL) of the samples (Table 10 and Fig. 2). Significant differences were noted in the % viability of all of the cells treated with the acetone extract and the compounds when compared to the maximum viability which was the control (0 µg/mL of samples). A significant cell decrease ($p< 0.001$) was noted in cancer cell lines treated with acetone extract (12.5–100 µg/mL) (Fig. 2). These results showed that, among the applications, the acetone extract contains mainly anacardic acids showed the high cytotoxic effect against the cancer cell lines, A549 and H1299 with IC_{50}=0.92 and IC_{50}=2.87 µg/mL, respectively and weaker cytotoxic effect against HUVEC healthy endotelial with IC_{50}=15.02 µg/mL (Table-10). However, as shown in Table-10, masticadienonic acid isolated from the acetone extract acted as a weaker cytotoxic agent as compared with the extract. These results concluded that the cytotoxic effect of the acetone extract is mostly due to the its anacardic acids components rather than masticadienonic acid. Likewise, masticadienonic acid is known to have antioxidant activity also induce apoptosis in colon [73,74], prostate [75] and human aortic endothelial cells [76] and inhibit cell proliferation and tumor growth in *in vivo* colorectal cancer modelling [77].

Shikimic acid isolated from the water extract was ineffective against HUVEC cells, but it acted as a weak cytotoxic against the cancer cell lines, whereas the water extract did not exhibit cytotoxic activity against all of the cell lines tested (Table 10 and Fig. 2). These results indicated that plant extracts and their pure metabolites may show different activities [78]. These results also pointed that this compound may be as a selective therapeutic agent against lung cancer cell lines. It has ben reported that shikimic acid regenerates human skin through converting human dermal fibroblasts [79] and protects skin cells from UV-induced senescence through activation of the NAD^+-dependent deacetylase SIRT1 [80]. It have also protective effect against cisplatin-induced renal injury [81] and hydroperoxide mediated oxidative stress [82]. Furthermore, shikimic acid promoted estrogen receptor positive breast cancer cells proliferation via activation of NF-κB signaling [83].



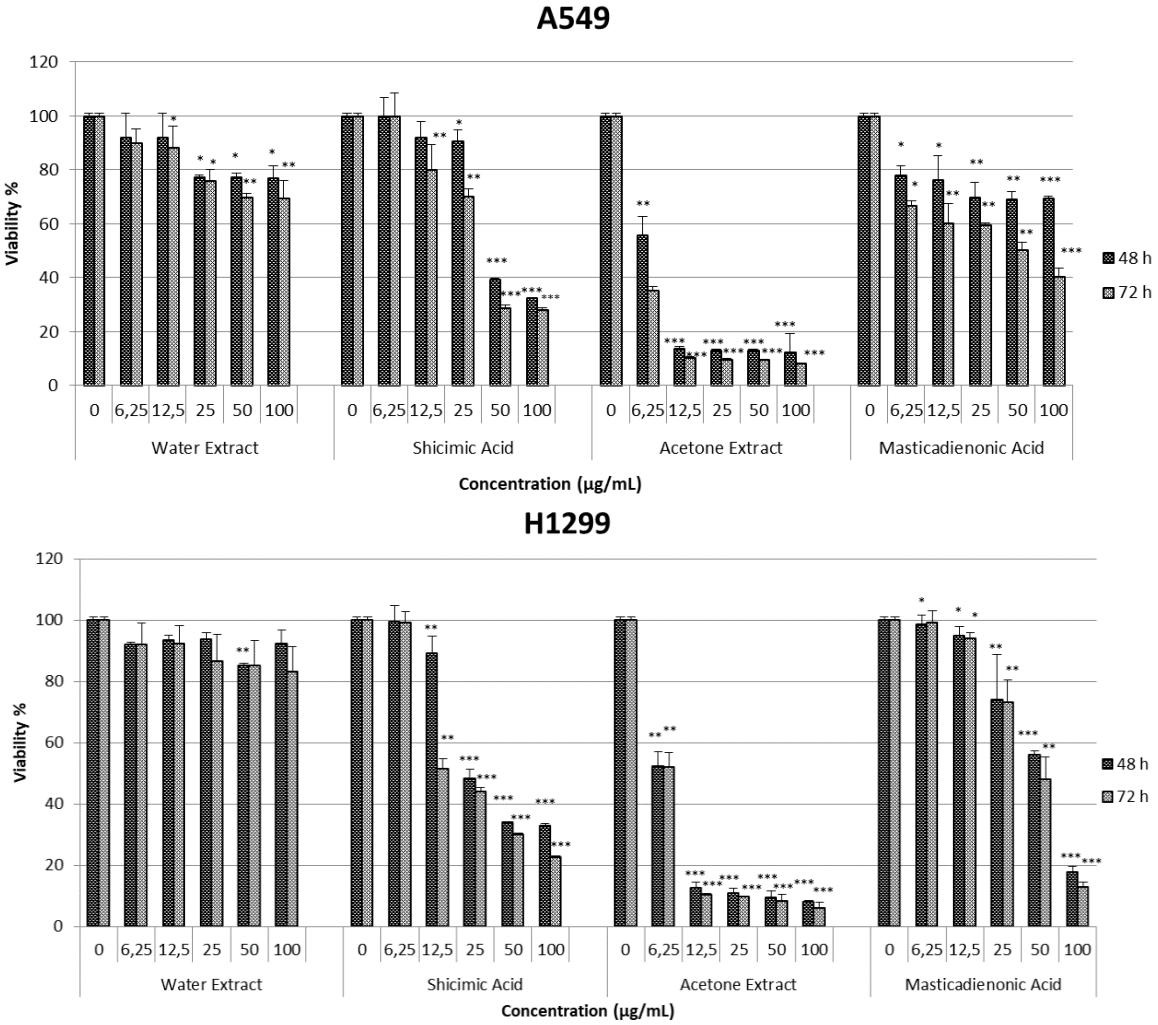


Fig. 2: Cytotoxicities of the extracts and the compounds on HUVEC, A549 and H1249 cells lines.

Table-10: Half-maximal inhibitory (IC_{50}) concentrations (µg/mL) of the extracts and the pure compounds of the soft hulls of pistachio fruits on the cells.

Treatments	HUVEC Duration of treatment		A549 Duration of treatment		H1299 Duration of treatment	
	48h	72h	48h	72h	48h	72h
Water extract	-	-	-	-	-	-
Shikimic acid	-	-	**43.24**	**39.67**	**30.12**	**18.82**
Acetone extract	**35.45**	**15.02**	**2.33**	**0.92**	**2.83**	**2.87**
Masticadienonic acid	**28.28**	**25.39**	-	**42.56**	**32.82**	**27.43**

Based on the present results, it can be concluded that the acetone extract of the soft hulls of pistachio fruits and its pure compounds have the potential to be used as chemotherapeutic drugs. The soft hulls of Pistachio fruits are known as an abundant source of bioactive phenolic compounds such as gallic acid, gallotannins and phenolic lipids, and anacardic acids [24, 25, 67, 84]. In accordance with our results, it has been reported that nonpolar organic solvents such as hexane, ethyl acetate, diethyl ether and acetone can be used for the selective extraction of anacardic acids in the mesocarps of pistachio fruits [25]. Anacardic acids is attracting increasing attention due to their anticancer [24,85,87], antioxidant [24,87], cardio-protective [88], and antibacterial [89] properties. Furthermore, the anacardic acids induces mitochondrial-mediated apoptosis in the A549 human lung adenocarcinoma cells [90,91]. These phenolics also modulates LPS-induced IL-8 expression [92] and induces ER stress and autophagy in a human alveolar epithelial cell line A549 [91].

Conclusion

The chemical constituents of the acetone extract of the mesocarp of *P. vera* fruits were investigated for the first time in the current study and it is rich in anacardic acid derivatives. Anacardic acids have been used in the production of an industrial liquid containg the cardanols known as CNSL (Cashew Nut Shell Liquid). Thus, a new source for cardanol production has been revealed with the current study [93]. Shikimic acid, mostly isolated from star anise with a yield of 8-9% is used as a starting molecule in the synthesis of an antiviral drug, oseltamivir used in the treatment of influenza. In this study, a new source for the production of shikimic acid was revealed and it was isolated from this source with a high yield (about 14%) [93]. The antidiabetic and anticholinesterase properties of the soft hulls of *P. vera* fruits were tested for the first time in this study against four different enzymes (α-glucosidase, α-amylase, AChE and BChE). The current results showed that the extract and the metabolites obtained from the soft hulls of pistachio fruits were very strong inhibitors on the activity of α-glucosidase and moderate or weak inhibitor on the α-amylase enzyme activity. These results suggest that both the acetone extract and its pure metabolites, the anacardic acids and its derivatives of *P. vera* fruits and soft hulls can be used as antidiabetic agents. In addition, this study showed that the extract obtained from the soft hulls of the pistachio fruits and its metabolites, masticadienonic and shikimic acids have cytotoxic activity against lung cancer. These results suggest that pistachio can also be used in therapeutic use. However, *in vivo* biological activities, safeties and toxicities of the acetone extract and its components should be demonstrated by further studies. The current results also show that the anacardic acids and its derivatives are selective and very strong BChE enzyme inhibitors. In the wiew of the present results, pure metabolites in the herbal extracts and herbal extracts can exhibit different pharmacological activities owing to the possible synergistic and/or antagonistic interactions.

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