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The *In Vitro* Impacts of Some Plant Extracts on Carbonic Anhydrase I, II and Paraoxonase-1

Karbonik Anhidraz I, II ve Paraoksonaz-1 Üzerine Bazı Bitki Özütlerinin *İn Vitro* Etkileri

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ABSTRACT

The presented article focuses on the in vitro inhibition of plant extracts on the human carbonic anhydrase isoforms (hCA I and hCAII), and paraoxonase-1 (PON1) activities. Five different plants (*Alcea rosea*, *Foeniculum vulgare*, *Elettaria cardamomum*, *Laurus azorica* and *Lavandula stoechas*) were selected in this study. Methanol, ethanol, and water extracts of plants were prepared and the concentration-dependent inhibition degrees were found for hCA I and hCA II isozymes and hPON1 spectrophotometrically. Thus, IC_{50} (mg/mL) values were obtained for each extract. Methanolic extract of *Elettaria cardamomum* has the highest inhibitory effects (0.032 mg/mL). The water extracts of plants showed lower inhibitory impacts compared to the methanol and ethanol extracts.

Keywords

Carbonic anhydrase, inhibition, paraoxonase, plant extract.

ÖZ

Bu makale insan karbonik anhidraz (CA) izoformları hCAI, hCAII ve paraoksonaz-1 (PON1) aktiviteleri üzerine bitki özütlerinin in vitro inhibisyonlarına odaklanmaktadır. Bu çalışmada beş farklı bitki (*Alcea rosea*, *Foeniculum vulgare*, *Elettaria cardamomum*, *Laurus azorica* and *Lavandula stoechas*) seçildi. Bitkilerin metanol, etanol ve su özütleri hazırlandı ve hCA I, II izoenzimleri ve hPON1 için derişime bağlı inhibisyon dereceleri spektrofotometrik olarak bulundu. Böylece her bir özüt için IC_{50} (mg/mL) değerleri belirlendi. *Elettaria cardamomum*'un metanol özütü en yüksek inhibisyon etkisine (0.032 mg/mL) sahipti. Bitkilerin su özütleri metanol ve etanol ekstreleri ile kıyaslandığında daha düşük inhibitor etkisi gösterdi.

Anahtar Kelimeler

Karbonik anhidraz, inhibisyon, paraoksonaz, bitki özütü.

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INTRODUCTION

Plant extracts have been applied for the treatment of many diseases in folk medicine such as diabetes [1] epilepsy [2], Alzheimer [3] and cardiovascular disease [4]. Besides the positive effects of these applications, the number of negative effects is not to be underestimated. Especially, plants are rich in terms of antioxidant components. Thus, they are widely consumed both as fresh food and extract on the world. Antioxidants are used to minimize the many diseases including cancer. Indeed, the source of many metabolic disorders is oxidative changes in the metabolism. The changes may have negative effects on the quality of life. Antioxidants are well known to role as preservatives against to oxidative stress. However, plants include various natural compounds in their structure. These compounds may be important for some disorders of living things. A lot of natural compounds may have still not been purified from plants. Therefore, use of the plant extracts has an important place as preservative or therapeutic in herbal medicine [5].

In the present study, *Alcea rosea (A. rosea), Foeniculum vulgare* Mill., *Cardamom (Elettaria cardamomum), Laurus azorica* L., *Lavandula stoechas* were selected as the source of the plant extract. *Alcea rosea (A. rosea)* (Family Malvaceae) commonly known as hollyhock which is a traditional Chinese herb [6]. The medicinal parts of *A. rosea* contain seeds, roots and flowers [7]. *A. rosea* flowers and roots have employed as febrifuge, diuretic, expectorant, demulcent, coolant, anti-inflammatory, and astringent agent [8]. *Foeniculum vulgare* Mill. (fennel) is a popular green aromatic herb of the family Apiaceae that is common throughout the Mediterranean region [9]. The essential oil of *Foeniculum vulgare* Mill. seeds (fennel oil) has been used to treat conditions of the reproductive, endocrine respiratory and digestive systems in traditional medicine [10]. Cardamom *(Elettaria cardamomum)*, commonly known as the "queen of spices" is one of the highly priced exotic spices in the world. Cardamom is mainly used for domestic culinary purposes and also has medicinal applications as a stimulant, digestive, breath freshener, carminative, and as an aphrodisiac. Cardamom has prominent antioxidant [11], anti-inflammatory [12], and antimicrobial properties [13]. *Laurus azorica* L., commonly known as bay leaves, belongs to Laureacea family, which *Laurus nobilis.* It is widely used as a spicy fragrance and flavor in traditional meat dishes, stews and rice [14]. *Lavandula stoechas* is a species of aromatic flowering plant of the Lamiaceae family [15]. It is used as cooking spices and fragrance, and its essential oil (EO) is found in the production of food, drinks, perfumes, cosmetics and pharmaceuticals [16-19].

It is well known that enzymes are crucial bio-catalyzers in the metabolism. Therefore, all substances taken in the body may interact with various enzymes. Especially some enzymes are called as drug-target and chemical target. Carbonic anhydrase and paraoxonase are known as drug-target enzymes in the literature. Carbonic anhydrases (CA, carbonate hydroliyase, E.C.4.2.1.1) are one of the most studied enzymes [20,21]. They are presented in all species and constitute a family involved in regulating pH and water, electrolyte, and ion transport [22]. The zinc-metalloenzymes play an important role in respiration, carbon dioxide and bicarbonate transport, pH and CO_2 balance in the lungs and tissues, the release of secretions from various tissues and organs, and especially in biosynthetic metabolic processes such as glycogenesis and lipogenesis in mammals [23]. Carbonic anhydrases are also necessary for CO_2 fixation in plants, algae, and prokaryotes. While the CA enzyme is common in organisms, its currently 16 isozymes are known in various tissues [24]. CA catalyzes the converting reaction from CO_2 to HCO_3^- which is named hydratase activity. It is a physiological activity of the CA.

On the other hand, CA enzyme exhibits also esterase activity under in vitro conditions. Inhibition or activation of CAs may important in terms of treatment many diseases such as diabetes, cancer, epilepsy, Alzheimer and cardiovascular disease. From this perspective, carbonic anhydrase inhibitors are clinically highly important compounds [25,26].

Paraoxonase (PON) is a mammalian lactonase that especially exists in the liver and HDL-bound enzyme. It is also known as drug target enzyme in the metabolism. Human PON1 (hPON1) is calcium-dependent enzyme. The enzyme consists two calcium ions its three-dimension structure. While one calcium ion is bound the active site, another is on structural part of the enzyme. PON1 consists of 355 amino acids having at least two N-linked carbohydrate chains. PON1 is synthesized in the liver and released from here into the blood [27]. Decrease of PON1 activities may related with some metabolic or genetic disorders such as arthritis, diabetes mellitus, rheumatoid cardiovascular diseases, age-related macular degeneration, chronic renal failure, and hyperthyroidism [27]. Moreover, PON1 has a significant place in HDL metabolism and in the prevention

of atherosclerosis. Some experimental data show that PON1 makes a central contribution to the antioxidant capacity of HDL Paraoxonase, by this antioxidant property prevent from oxidation of both HDL and LDL. Thus, there is a close physiological relation between PON1 and HDL in plasma [28]. HDL facilitates the secretion of the PON1 from the liver, stabilizes the enzyme and provides the hydrophobic environment that is needed for the function of PON1 [29]. It is now known that the effects of chemicals on enzymatic mechanisms are important. Because, almost all reactions in the metabolism are catalyzed by various enzymes. These dramatic changes on the enzymatic mechanisms may lead to many disorders as mentioned above. Therefore, to determine the impacts on enzyme activities of herbal extracts commonly used in alternative medicine is crucial.

The present article focuses on in vitro inhibitory effects on human PON1 and CA I and II isoforms of *Alcea rosea, Foeniculum vulgare, Elettaria cardamomum, Laurus azorica* and *Lavandula stoechas.*

MATERIALS and METHODS

Materials

Paraoxon, p-Nitrophenylacetate, Cyanogen bromide-activated-Sepharose 4B, protein assay reagents and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. (Sigma- Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were analytical grade and obtained from Merck (Merck KGaA Frankfurter Strasse 250, D 64293 Darmstadt, Germany).

Plant material and Extraction

Plants were obtained from a local market at Erzurum, Turkey. Extraction process was done according to previous study [30].

Purification of Carbonic Anhydrase Isozymes and Esterase Activity Assay

CA isoforms were purified using Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography in a single step [31]. The affinity material, Sepharose-4B-L-tyrosine-sulfanilamide, was prepared conforming to our previous technique [31]. The protein eluates were measured at 280 nm as described previously, spectrophotometrically [32,33]. The activities of CA isoforms were evaluated according to the Verpoorte et al. (1967) [34]. p-Nitrophenylacetate was used as a substrate converted by both isoforms to the p-nitrophenolate ion in this technique. The absorbance changes were determined during 3 min at 25°C.

Protein Quantity Assay

Bradford procedure was used for determination of protein amount [35]. The bovine serum albumin was used as standard for this evaluation, which was done at 595 nm according to previous studies [36-38].

SDS-Polyacrylamide Gel Electrophoresis

According to Laemmli's procedure (1970) the purity and presence of carbonic anhydrase isoforms were observed by the SDS-PAGE technique [39]. The method was performed according to our previous studies [29,40,41]. After this procedure, a single band was seen for each isoform (Figure 1). Molecular weight of the enzyme was determined by using SynGene imaging tool (Figure 2).

Ammonium Sulfate Precipitation of PON1

20 ml of human serum was precipitated with solid ammonium sulfate at 60-80%., Centrifugation was carried out at 24000xg for 20 minutes during each precipitation. The pellet was dissolved in 100 mM Na-phosphate buffer pH 7.0 and dialyzed against same buffer [28].

Measurement of PON1 Activity

hPON1 activity was measured using paraoxon (diethyl p-nitrophenyl phosphate) as substrate (1 mM) in 50 mM glycine/NaOH (pH 10.5) including 1 mM $CaCl_2$. hPON1 assay was based on the measurement of p-nitrophenol at 412 nm [42].

RESULTS and DISCUSSION

Plants are employed for the treatment of many diseases date back to prehistory and all people have this ancient tradition. Due to their antioxidant, antimicrobial, antifungal antitumoral and anti-inflammatory activities, natural products have been used for a long time. Nowadays, plant extracts are consumed as health preservative materials among the people. This rate is almost over 80% of the world's population [43]. Most of the plants are rich in terms of phenolic compounds, benzophenones, xanthenes, bioflavonoid, flavonoid, terpenes as well as some metabolites such as anthraquinones, cyanates, oxalate, tannins and saponins [44]. Supportive roles of these compounds are indisputable in the living metabolism.

In this study, ethanol, methanol and water extractions of *Alcea rosea, Foeniculum vulgare, Elettaria cardamomum,*

Laurus azorica and *Lavandula stoechas* were prepared and measured effects of these samples against purified hPON1, hCA I and hCA II. For this reason, cytoplasmic CA isoforms, hCA I and hCA II, were purified from human erythrocytes with a simple and one step affinity method. Throughout the purification steps, CA-I was obtained with a specific activity of 768.5 U/mg proteins, with a yield of 62.25%. CA-II was also obtained with a specific activity of 930.34 U/mg proteins, with a yield of 23.27%. It is shown in Table 1.

The purity and molecular weight of the enzymes were determined approximately 29 kDa using the SDS-PAGE. (Figure 1 and Figure 2).

PON1 enzyme was obtained from human serum by only ammonium sulfate precipitation. Subsequently, ethanol, methanol and water extractions of the plant extracts were tested on purified enzyme activities. Almost all extracts inhibited the enzymes. It is important, because both CAs and PON1 enzyme have esterase activity and the same reaction product is obtained at the end of the reaction (Figure 3).

Alcea rosea (A.rosea) is used as herbal remedy in folk medicine for treatment of different diseases such as inflammation of the kidneys and the uterus, kidney and urinary tract infections, malaria, rheumatism [45]. Some pharmacological effects including antibacterial, analgesic, anti-inflammatory and cytotoxic activities have been reported [46]. The inhibition results are expressed as IC_{50} (mg/mL) in the present study. As appeared from Table 1, hCAI, II and hPON1 activities are inhibited by *Alcea rosea* extracts. Only water extract did not have any inhibition or activation effect. Methanol extract was most effective for both hCAI and II. On the other hand, ethanol extract of *Alcea rosea* inhibited the hPON1 enzyme activity, strongly (0.074 mg/mL) (Table 2). There are some researches about the inhibition effects of *Alcea rosea* extracts on various

Table 1. Purification steps of hCA isoforms from human erythrocytes.

Step	Activity (EU/mg)	Protein (mg/mL)	Volume (mL)	Total Activity (EU)	Total Protein (mg)	Specific Activity (EU/mg)	Recovery (%)	Purification Fold
Hemolysate	130.0	4.60	38	4940.0	174.8	28.2	100	1
Sepharose-4B-L tyrosine-sulfanilamide affinity chromatography and dialysis for hCAI	416.6	0.542	7.5	3124.5	4.06	768.5	63.25	27.25
Sepharose-4B-L tyrosine-sulfanilamide affinity chromatography and dialysis for hCAII	383.3	0.412	3.0	1149.9	1.236	930.34	23.27	32.99

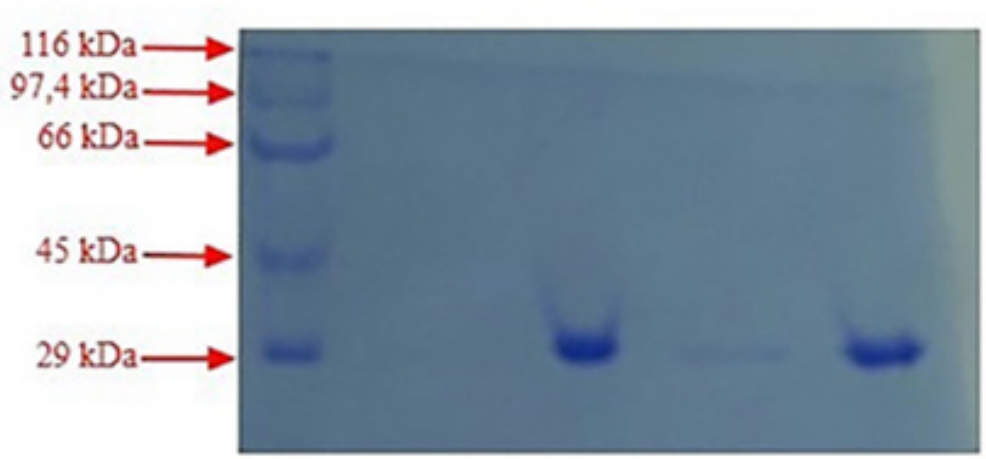

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis of purified hCA isoforms. Lane 1: standard proteins, Lane 2: purified hCA-I, Lane 3: purified hCA-II.

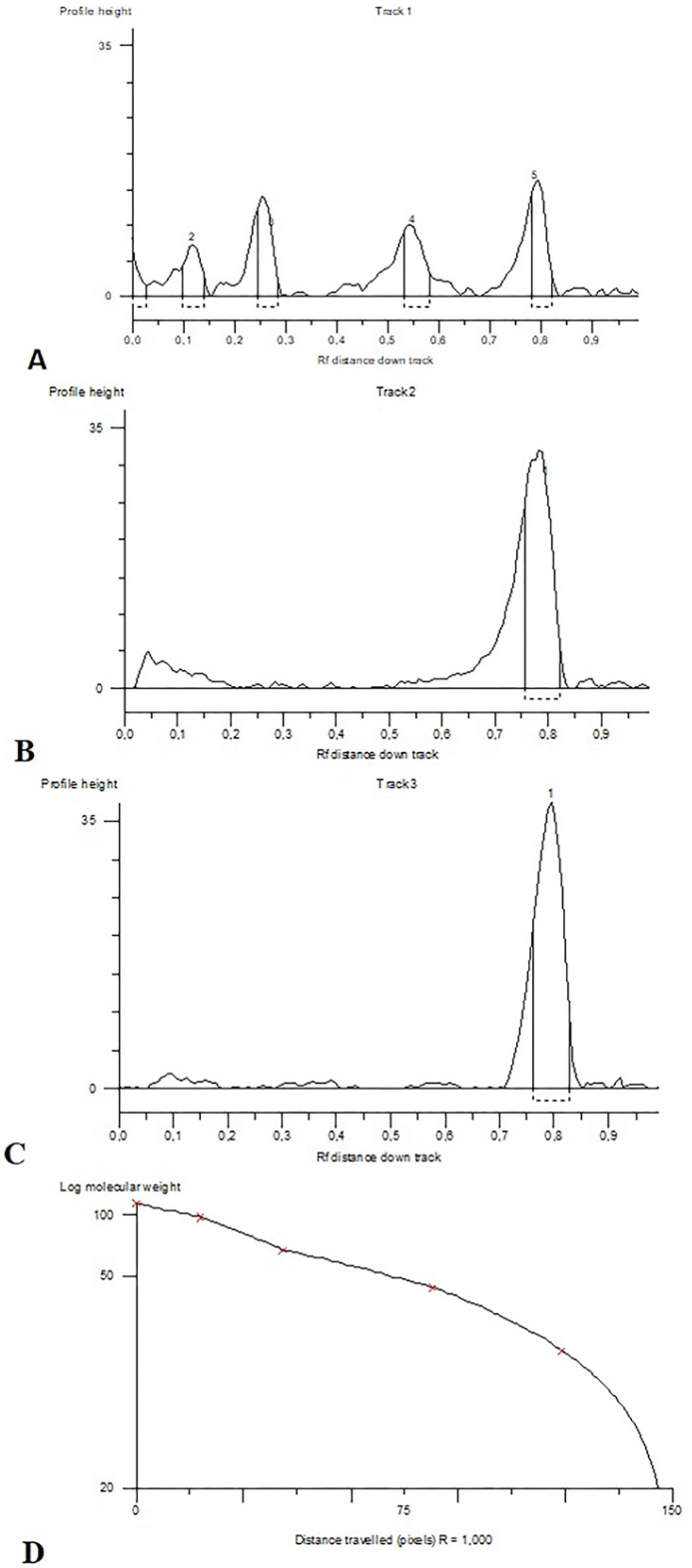

Figure 2. Molecular weight of the hCA I and hCA II isozymes were calculated about 29 kDa by using SynGene imaging tool. Track 1 is the peaks of standard proteins. Track 2 is CA I's peak, and Track 3 is CA II's peak.

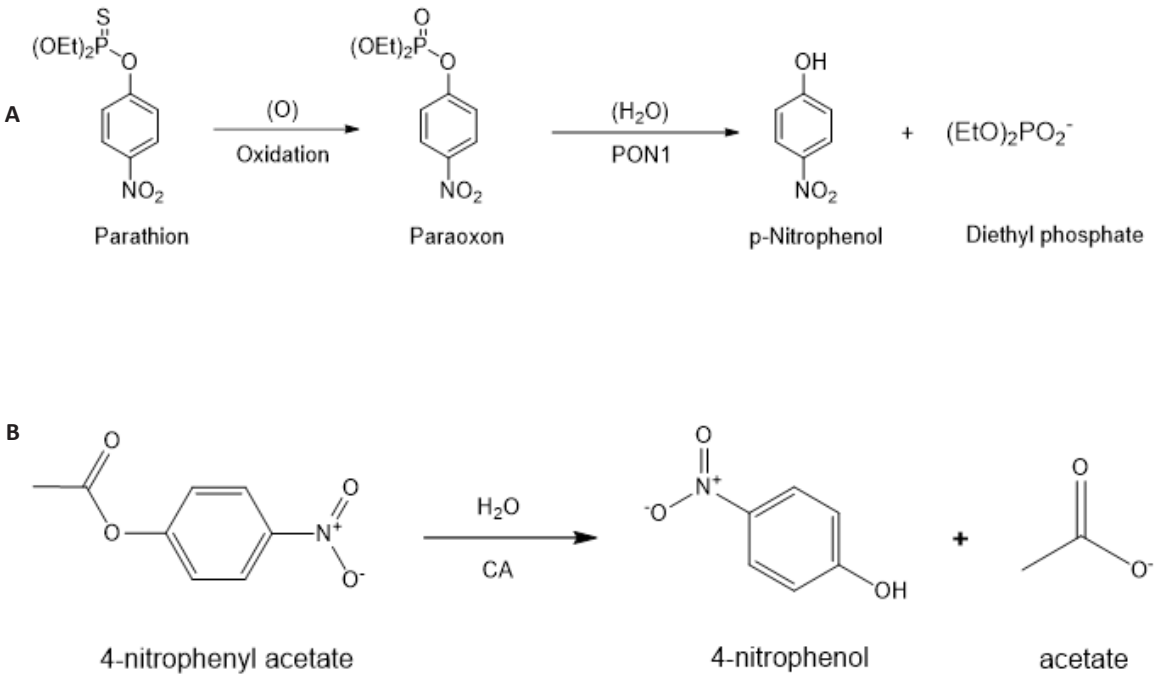

Figure 3. A) Mechanism of paraoxonase activity assay B) Mechanism of carbonic anhydrase esterase activity assay.

Table 2. Inhibitory effects of *Alcea rosea, Foeniculum vulgare, Elettaria cardamomum, Laurus azorica* and *Lavandula stoechas* different extracts against hCA I, hCA II and hPON1.

Plant Extracts	Solvent	hCA I IC_{50} (mg/mL)	hCA II IC_{50} (mg/mL)	hPON1 IC_{50} (mg/mL)
Alcea rosea	Water	---	1.270	1.721
	Ethanol	0.130	0.119	0.046
	Methanol	0.074	0.077	0.295
Foeniculum vulgare	Water	0.301	---	1.60
	Ethanol	---	0.162	0.073
	Methanol	0.183	0.129	0.200
Elettaria cardamomum	Water	0.991	1.400	1.361
	Ethanol	0.065	0.129	0.054
	Methanol	0.032	0.061	0.094
Laurus azorica	Water	0.842	1.170	0.732
	Ethanol	0.692	0.785	0.060
	Methanol	0.458	0.472	0.080
Lavandula stoechas	Water	0.119	0.330	---
	Ethanol	0.077	0.067	0.047
	Methanol	0.080	0.054	0.092

enzymes. For instance, Namjoyan et al. (2015) investigated the effects on diphenolase activity of mushroom tyrosinase of some plant extracts including Alcea rosea [47]. They found the IC_{50} value for *Alcea rosea* as 2.82 mg/mL.

Foeniculum vulgare is widespread in northern Anatolia. While the water extract did not have any effect on hCA II isozyme, ethanol extract did not have any effect on hCA I isozyme. Interestingly, hPON1 which is HDL-related antioxidant enzyme having an important role in the prevention of cardiovascular, was inhibited by each extract, and particularly, the enzyme activity was more decreased via ethanol extract (IC_{50} 0.073 mg/mL) Results were shown in Table 2.

Indeed, it is known that *F. vulgare* is a significant source of phenolic acids including of 1,4-O-di-caffeoylquinic acid, 1,3-O-di-caffeoylquinic acid, 1,5-O-di-caffeoylquinic acid, 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, the flavonoids like rosmarinic, quercetin-3-rutinoside and eriodictyol-7-rutinoside [48]. They are phenolic compounds as well as antioxidant properties. For all this, we estimate that in our study, other factors in the content of the plant extract may be effective for inhibition of PON1 enzyme apart from phenolic compounds. In another study, thirteen compounds were isolated from a methanol extract of *F. vulgare* and tested for their inhibition on CYP3A4. 5-Methoxypsoralen (5-MOP) was seen to have strongest inhibition with IC_{50} value of 18.3 µM and a mixed type of inhibition [49].

Elettaria cardamomum (L.), cardamom, is known as small cardamom and discriminated from large cardamom (*Amonum subulatum Roxb.*). Its oil has been employed in a wide range of beauty products [50]. Water, ethanol and methanol extracts of *Elettaria cardamomum* (L.) inhibited all enzymes. However, IC_{50} values of methanol extract were lower than other extract for hCAI and II isozymes. On the other hand, ethanol extract was seen to stronger inhibit the PON1 enzyme than other ones (Table 2; Figure 4).

Also, while IC_{50} values of hCA I and II obtained for methanol extracts of *Laurus azorica* was low, the highest inhibition for hPON1 was in ethanol extract (Table 2). The results were similar to *Laurus azorica* for *Lavandula stoechas* etracts (Table 2). There are some studies about the effects on enzymes of *L. Stoechas* extract. For example, Carrasco et al. (2015) investigated *L. Stoechas* extract as an enzyme target [51]. They prepared three samples of *L. Stoechas* and studied the effects of samples on lipoxygenase (LOX) activity. They determined the inhibition for LOX activity, significantly.

CONCLUSION

Carbonic anhydrase I and II isozymes were purified from human erythrocytes at one step by simple affinity technique. Paraoxonase 1 enzyme was obtained from human serum with partial purification using ammonium sulfate precipitation. Subsequently, water, ethanol and methanol extracts of *Alcea rosea, Foeniculum vulgare, Elettaria cardamomum, Laurus azorica* and *Lavandula*

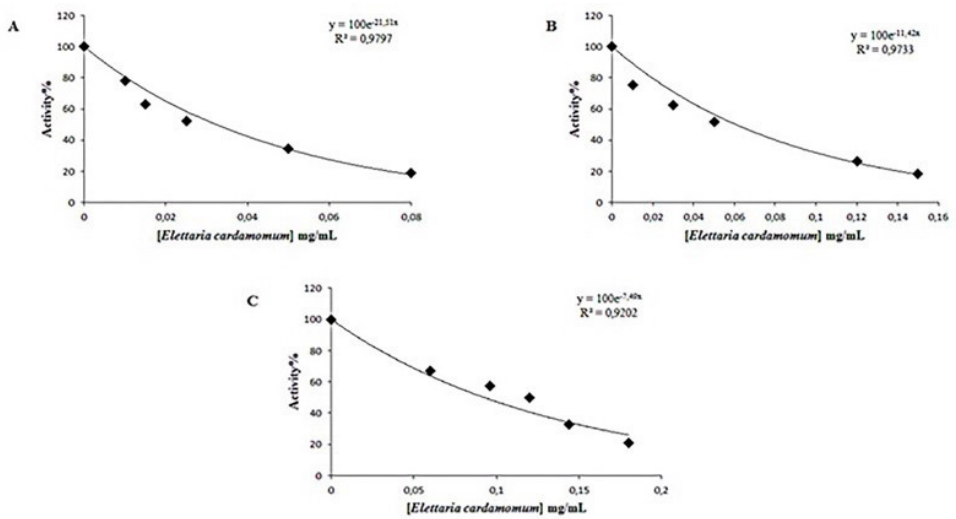

Figure 4. IC_{50} graphs of the methanolic extracts of *E. cardamomum*. A) For carbonic anhydrase I B) For carbonic anhydrase II C) For paraoxonase enzyme activity.

stoechas were tested on hCA I, II and PON1 enzymes. IC_{50} values were determined as mg/ml for each extract. According to the results, highest inhibition values were obtained in particular ethanol and methanol extracts of *Elettaria cardamomum*. As known, inhibition of the enzymes was crucial in the treatment of many diseases. However, using of the various plant extracts may lead to the formation some aggregates in the metabolism. Therefore, the next step of the study should be to determine the inhibitors containing extracts.

Acknowledgments

The authors declare that they have no conflict of interests.

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