Research Article GU J Sci 38(1): 65-73 (2025) DOI: 10.35378/gujs.1416864

Gazi University

Journal of Science

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The Effects of Anatolian Black Pine Cone Extracts on Carbonic Anhydrase Enzymes

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Highlights

- Anatolian black pine cone extracts were prepared using solvents of different polarities.
- The effects of the extracts on hCA I and hCA II activities were examined.
- The extracts had remarkable inhibition effects on hCA I and hCA II.

Article Info

Received: 09 Jan 2024
Accepted: 13 Aug 2024

Keywords

Anatolian black pine
Cone
Carbonic anhydrase
Esterase activity
Inhibition

Abstract

Nowadays, there is an increasing trend in the use of natural products to treat diseases or alleviate their effects. This situation has emerged as a result of the side effect problem seen in the use of synthetic drugs. In this study, Anatolian black pine cone extracts were prepared using solvents of different polarities (water, methanol, ethanol, ethyl acetate, *n*-hexane). Then, the effects of these extracts on the esterase activities of hCA I and hCA II isoenzymes, which are associated with many physiological disorders, were examined *in vitro*. IC_{50} values were in the range of 5.10 to 49.50 µg/mL for hCA I and 2.57 to 15.07 µg/mL for hCA II. It can be said that these results are promising in terms of the potential of Anatolian black pine cone as a natural pharmacological agent.

1. INTRODUCTION

Meldrum and Roughton, who were investigating the transport of gases in the blood of vertebrates, discovered carbonic anhydrase (CA), which catalyzes the reversible hydrolysis of carbon dioxide (CO_2) and water (H_2O) to bicarbonate (HCO_3^-) and proton (H^+), in 1933 [1]. The reaction of two simple molecules (CO_2 and H_2O) produces a weak base (HCO_3^-) and a strong acid (H^+), which induces a pH change [2]. This system is used as an effective means of pH regulation by many organisms from Prokaryotes to Eukaryotes [3]. To date, eight genetically different CA families (α-, β-, γ-, δ-, ζ-, η-, θ- and ι-) have been defined [4-8]. Mammalian CAs, which belong to the class of α-CAs, have 16 different isoenzymes in mammals. As in all organisms encoding CA enzymes, human CA enzymes (hCAs) play a role not only in pH regulation but also respiration, CO_2 and HCO_3^- transport, electrolyte secretion, pH and CO_2 homeostasis, gluconeogenesis, adipogenesis, ureagenesis, bone resorption and calcification [3,9-11]. It is inevitable that CA isoenzymes, which are involved in many processes, are associated with multitude of diseases. Glaucoma, retinal and cerebral edema, epilepsy, obesity, hypoxic tumors, inflammation, neuropathic pain, Alzheimer, oxidative stress and vertigo are diseases associated with hCA isoenzymes [12-14]. Interfering with the activities of hCAs using modulators (activators or inhibitors) is pharmacologically important for the management of CA-related diseases. Despite tremendous strides over the past decade to understand the molecular interactions associated with CAs, the development of selective CA inhibitors (CAIs) is still an ongoing process. Over the past years, various research groups have worked to design and synthesize new CAIs effective against a variety of pathological conditions in which the activity of these enzymes is dysregulated. Sulfonamides, phenols, sulfamates, sulfamides, coumarins, polyamines, dithiocarbamates,

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xanthates, benzoxaboroles, phosphonamidates, selenols, and ninhydrins are classes of CAIs discovered from 1940 to 2020 [9]. Compounds of completely natural origin as well as synthetic or natural-synthetic hybrid CAIs have been studied as CAIs. In the literature, there are studies in which CAIs of natural origin are used directly as extracts and the compounds obtained from the extract by chromatographic techniques are examined as CAIs. Some plants in which hCA inhibition effects were investigated as total extracts can be listed as *Foeniculum vulgare* Mill., *Alcea rosea* L., *Laurus azorica* (Seub.) Franco, *Elettaria cardamomum* L., *Lavandula stoechas* L. [15], *Artemisia dracunculus* L. [16], *Leucas cephalotes* (Roth) Spreng. [17], and *Abrus precatorius* L [18].

Anatolian black pine (*Pinus nigra* Arnold.) is distributed in the Balkans, Southern Carpathians, Crimea, Turkey, Cyprus and Syria. It has a smooth cylindrical body that can reach up to 40 m in length and exceed 1 m in diameter. Needle leaves are 4–8 cm long, dark green and hard. The ovoid-conical cones are symmetrical and have almost non-existent short stems. Cone length varies between 3.5 cm – 10 cm [19]. The production of pine cone extracts, whose medicinal and protective properties have been discovered, has gained importance in recent years. It has been reported that some pine cone extracts have toxic activities against fungi. It has also been observed that essential oils obtained from pine cones protect the plant against fungal and insect attacks. The cones, leaves and resins of the *Pinaceae* family are believed to have healing effects among the public and are used in the treatment of various disorders [20]. In East Asia, cones, cortex, needles, and pollen, which are various parts of pine trees, are widely consumed as dietary supplements to support immune system. In these countries, it is believed that consumption of pine tree fractions positively affects neuronal and gastrointestinal problems and prevents diabetes, hypertension and atherosclerosis. In studies conducted on the use of antioxidant components found in the structure of pine bark and cones in foods, it has been proven that the present components prevent protein oxidation at rates ranging from 42% to 64%. In traditional medicine, pine cones have been used to relieve cough, moisten the lungs and reduce fever [21,22].

In last two decades, there has been an increase in the use of natural compounds/extracts instead of synthetic compounds for the treatment or prevention of diseases, as they have fewer possible side effects. In this context, various groups have studied the CA inhibition effects of some of the extracts mentioned above. In this study, extracts of pine cones were prepared using solvents of different polarities (water, methanol, ethanol, ethyl acetate and hexane). The inhibitory effects of the prepared extracts on hCA I and hCA II isoenzymes were determined as *in vitro*.

2. MATERIAL METHOD

The pine cones used in the study were collected from Anatolian black pine trees in Kütahya Dumlupınar University Evliya Çelebi Campus (Türkiye). Extraction solvents (methanol, ethanol, ethyl acetate and *n*-hexane) were purchased from Fischer Scientific International Inc. (New Hampshire, USA). TRIS and H_2SO_4 used to prepare buffer solution were purchased from Merck (Darmstadt, Germany). 4-nitrophenyl acetate, which was used as a substrate in kinetic measurements, was purchased from Sigma-Aldrich (Missouri, USA). DMSO, which was used for preparation of stock inhibitor solutions, was purchased from Fischer Scientific International Inc. (New Hampshire, USA). Enzyme activity measurements were performed with SHIMADZU UV-1700 PharmaSpec UV-Vis spectrophotometer (Kyoto, Japan). Buffer solutions were prepared with the pH meter (Mettler Toledo SevenDirect SD-20 Kit, Ohio, USA). Grinding operations were carried out using an IKA A11 basic grinder (Staufen, Germany).

2.1. Preparation of Pinecone Extracts

The unopened cones collected from *Pinus nigra* Arnold. were kept in a sunless environment at room temperature for a few days. 50 mL of solvent (water, methanol, ethanol, ethyl acetate and *n*-hexane) was added to 5 g of the sample taken from the milled pinecone and mixed for 24 h at 25 °C. The suspension was filtered and the filtrate was centrifuged at 10^5 *g* for 20 min to obtain clear solution. Then the supernatant was concentrated at low temperature and low pressure in a rotary evaporator. The solid residue was weighed and stored in -20 °C until used [15,16,23,24].

2.2. Purification of hCA I and hCA II

The hCA I and hCA II isozymes were purified from erythrocytes by Sepharose®4B-L-tyrosine-*p*-aminobenzenesulfonamide affinity chromatography, where Sepharose®4B is the matrix, L-tyrosine is the spacer arm, and *p*-aminobenzenesulfonamide is the ligand of the affinity column. The purity of the isozymes was checked by SDS-PAGE, and the protein quantity of the eluates was determined according to the Bradford method. The purification procedure has been described in detail in our previous studies [25-29].

2.3. Enzyme Activity Assay

All kinetic studies were performed based on the esterase activities of hCA isozymes (Figure 1). Esterase activity was determined by measuring the absorbance change occurring during the hCA-catalyzed hydrolysis reaction of 4-nitrophenyl acetate substrate to 4-nitrophenolate over a period of 3 min (assay conditions: 348 nm, 25 °C). The assay mixture has contained 0.05 M Tris (pH 7.4), 3 mM 4-nitrophenyl acetate, and hCA solution in a total volume of 3.0 mL. A reference measurement was obtained without enzyme solution [30].

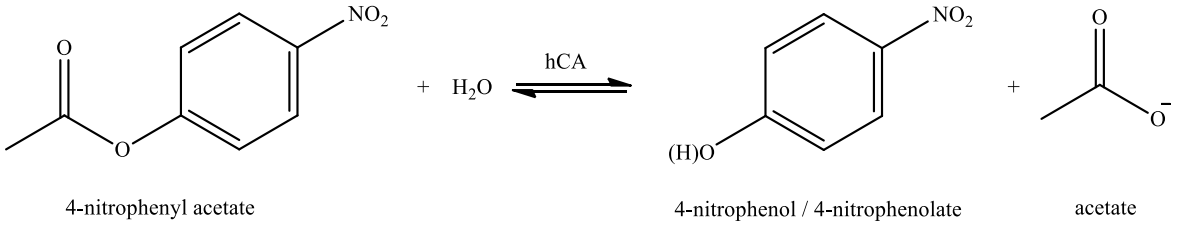

Figure 1. *Ester hydrolysis reaction catalyzed by hCA enzymes*

2.4. *In vitro* Inhibition Studies

In order to use the extracts obtained from pine cones in inhibition studies, stock solutions were prepared in DMSO at a concentration of 0.1 mg/mL. To determine the IC_{50} values of the extracts, esterase activities of hCA isoenzymes were analyzed in the presence of extract solutions. The regression analysis graphs were drawn by plotting relative enzyme activity (%) vs. inhibitor concentration ($\mu g.mL^{-1}$) using the Microsoft Excel Package Program (Microsoft Office 2016), and IC_{50} values were calculated. All of the presented results were confirmed in three independent experiments and were expressed as the mean ± standard deviation [31-33].

3. RESULTS AND DISCUSSION

In this study, the effects of extracts prepared from Anatolian black pine cones using different solvents on CA enzymes, which are associated with many physiological and pathological disorders such as glaucoma, obesity, vertigo, epilepsy, altitude sickness, retinal/cerebral edema, infertility, cariogenesis, oxidative stress, stroke, retinopathy and cancer, were examined as *in vitro*. hCA I and hCA II were chosen as target isoenzymes for determination of pharmacological properties of pine cone extracts. Because these isoenzymes are associated with diseases such as retinal/cerebral edema, glaucoma, altitude sickness, epilepsy and vertigo. In addition, the high expression of these isoenzymes in erythrocyte cells allows them to be purified more easily.

hCA I and hCA II isoenzymes were isolated in high purity from erythrocyte cells in a single step by affinity chromatography. The hCA I isoenzyme was purified 132.25-fold with 15.75% yield and 995.86 EU/mg protein specific activity, while hCA II was purified 251.16-fold with 22.32% yield and 1618.42 EU/mg protein specific activity. After the pine cones were ground and ready for extraction, they were extracted with solvents of different polarities. The reason why solvents of different polarities are chosen for extraction is that there are many components with different molecular structures in natural materials. Therefore, a wide polarity scale allows substances with different properties to be extracted. Another reason for using different extraction solvents is the differences of active site structure of hCA isoenzymes. Due to the

catalysis mechanism of these isoenzymes, approximately half of the active site consists of hydrophobic amino acid residues, while the other half consists of hydrophilic amino acid residues. Therefore, since substances of different polarities are obtained in extraction processes with solvents of different polarities, the possibility of obtaining extracts that can inhibit hCA isoenzymes has increased.

Table 1. *Amount of the extracts obtained from larch pinecones*

Solvent	Extract amount (g)
Water	0.82
Methanol	0.61
Ethanol	0.42
Ethyl acetate	0.27
n-Hexane	0.13

The best extraction efficiency was obtained in the presence of water, which is the most polar solvent. As expected, this is due to the polyphenols present in large amounts in pinecones. As the polarity decreased, the amount of extract obtained also decreased (Table 1). This shows that the oily substances in the pinecone are in very small amounts. The reason why DMSO was chosen as the extract solvent for inhibition studies is that it can dissolve all extracts and does not inhibit or activate CA enzymes.

Table 2. *Esterase IC_{50} values of the Pinus nigra pinecone extracts for hCA isoenzymes*

Extracts	IC_{50} ($\mu g.mL^{-1}$)	
	hCA I	hCA II
Acetazolamide[a]	1.652±0.033	0.016±0.0001
Water	11.18±0.56	7.37±0.37
Methanol	5.10±0.26	2.57±0.13
Ethanol	12.60±0.63	10.19±0.51
Ethyl acetate	30.13±1.51	11.36±0.57
n-Hexane	49.50±2.48	15.07±0.75

[a]Control compound, and the IC_{50} values of this compound were taken from [16]

Among the extracts used in the study, methanolic extract had the strongest inhibitory effect (hCA I IC_{50} value is 5.10 µg/mL, and hCA II IC_{50} value is 2.57 µg/mL) (Figure 2). However, it is important for a pharmacological agent to be selective among CA isoenzymes as well as being a strong CA inhibitor. When the IC_{50} values of the extracts are examined, it is seen that all of them are relatively more selective against the hCA II isoenzyme (Table 2).

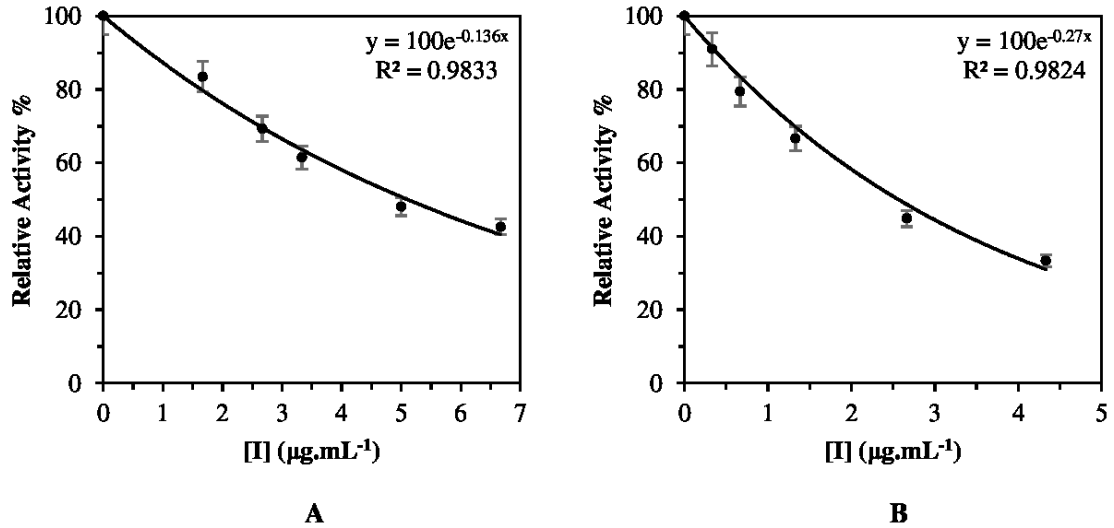

Figure 2. *Enzyme activity vs. inhibitor concentration graphs of methanolic extract (**A:** hCA I, **B:** hCA II)*

When the amino acid sequences in active sites of hCA I and hCA II are compared, it is seen that there are differences mostly between the amino acid residues in the hydrophobic part (Table 3) [34]. It is possible, then, that these differences enable the extracts to interact more tightly with hCA II. The fact that the extract prepared using *n*-hexane inhibited the hCA II isoenzyme approximately 3.5 times more strongly than hCA I indicates that the selectivity may be due to the hydrophobic part of the isoenzymes. However, to make a definitive comment, the compounds in the extract should be determined, and molecular docking studies and even X-ray diffraction analyses should be performed (Figure 3).

Table 3. *Hydrophobic amino acid residues in the active sites of hCA I and hCA II*[*]

Residue	hCA I	hCA II
91	Phe	Ile
121	Ala	Val
131	Leu	Phe
135	Ala	Val
141	Leu	Leu
143	Val	Val
204	Tyr	Leu
207	Val	Val
209	Trp	Trp

[*] Data was taken from [34]

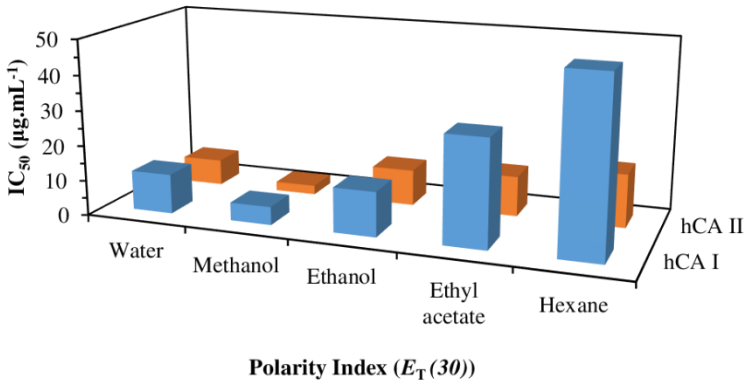

Figure 3. *IC_{50} values and isoenzyme selectivities according to extract polarity (E_T (30) values were taken from [35])*

Some studies in the literature on the subject and the results obtained are explained below. Kaya et al. (2019) prepared aqueous, ethanolic and methanolic extracts of *Foeniculum vulgare*, *Elettaria cardamomum*, *Alcea rosea*, *Lavandula stoechas* and *Laurus azorica* plants and examined their inhibition effects on hCA I and hCA II in terms of esterase activity inhibition. They determined that methanolic extracts of all plants were the most effective CA inhibitors [15]. In a study in which dichloromethane, *n*-hexane, methanol and ethanol extracts of *Artemisia dracunculus* L. leaves were prepared and their inhibition effects on hCA I and hCA II esterase activity were examined, it was determined that dichloromethane extract was the strongest inhibitor (hCA I IC_{50} value was 0.020 µg/mL) [16]. In another paper, methanol and oil extracts of *Cucumis melo* L. seeds were reported to show interesting biological activities for human erythrocyte CAs. While these extracts activated the hCA I, they inhibited the hCA II isoform. hCA II IC_{50} values were determined as 0.497 ng/mL for the oil extract and 10.98 µg/mL for the methanol extract [36]. Aydin et al. (2021) prepared extracts of the *Satureja cuneifola* plant with petroleum ether, chloroform and methanol, examined their effects on hCA I and II esterase activities, and found that the methanol extract showed the strongest inhibitory effect. Researchers had determined the hCA I esterase IC_{50} value of the direct methanol extract as 31 µg/mL, and the hCA II IC_{50} value of the methanol extract as 12 µg/mL [37].

The IC_{50} values determined in our study were in the range of 5.10 – 49.50 µg/mL for hCA I and 2.57 – 15.07 µg/mL for hCA II (Table 2) and were comparable to other studies in the literature. Additionally,

similar to some studies in the literature, the strongest inhibitory effect was observed in the methanolic extract in our study.

4. CONCLUSION

In recent years, the use of natural compounds both as food supplements and for therapeutic purposes has become widespread. Intensive efforts are being made to discover therapeutic agents of natural origin. Within the scope of this study, the effects of Anatolian black pine cone extracts, whose many benefits are known to the public and reported in the literature, on hCA I and hCA II isoenzymes, which are known to be associated with various disorders, were examined *in vitro*. While methanolic extract showed the strongest inhibitory effect among the extracts, *n*-hexane extract showed the most selective inhibitory effect on hCA II isoenzyme. These promising results should be supported by further studies including *in vivo* tests and toxicity tests.

ACKNOWLEDGEMENTS

This study was supported by TUBITAK (grant number 1919B012105233).

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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