

## Investigation of The Effects of Some Edible Mushroom Extracts on Human Carbonic Anhydrase Isozymes

### Bazı Yenebilir Mantar Ekstrelerinin İnsan Karbonik Anhidraz İzozimleri Üzerine Etkilerinin İncelenmesi

Research Article

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#### ABSTRACT

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In this study the effects of four edible mushroom species (*Lentinula edodes*, *Lactarius deliciosus*, *Lactarius deterrimus*, *Terfezia boudieri*) on human carbonic anhydrase isozymes (hCA I and hCA II) were investigated as in vitro. hCA I and II isozymes were purified from erythrocytes by using Sepharose®4B-L-tyrosine-*p*-aminobenzene sulfonamide affinity chromatography. Later the effects of the mushroom extracts on the hydratase and esterase activities of hCA I and II were measured. *L. edodes* showed inhibitory effect on the esterase activities of hCA I and II. However, other extracts showed activator effects on the esterase activities of hCA I and II.

#### Key Words

Carbonic anhydrase, hydratase activity, esterase activity, mushroom extracts.

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#### ÖZET

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Bu çalışmada, dört yenebilir mantar türünün (*Lentinula edodes*, *Lactarius deliciosus*, *Lactarius deterrimus*, *Terfezia boudieri*) insan karbonik anhidraz izoenzimleri (hCA I ve hCA II) üzerine etkileri in vitro olarak incelenmiştir. hCA I ve II izoenzimleri Sepharose®4B-L-tirozin-*p*-aminobenzen sülfonamid afinite kromatografisi kullanılarak eritrositlerden saflaştırıldı. Daha sonra mantar özütlerinin hCA I ve II izoenzimlerinin hidrataz ve esteraz aktivitelerinin üzerine etkileri ölçüldü. *L. edodes* hCA I ve II nin esteraz aktiviteleri üzerinde inhibitör etki göstermiştir. Ancak diğer özütler hCA I ve II nin esteraz aktiviteleri üzerinde aktivatör etki göstermişlerdir.

#### Anahtar Kelimeler

Karbonik anhidraz, hidrataz aktivitesi, esteraz aktivitesi, mantar özütleri.

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## INTRODUCTION

Mushrooms have been used as popular food materials in many cultures for centuries [1,2]. Several mushroom species have been consumed as alternative therapeutic agents in the prevention of certain diseases, as well as their nutrient values [3]. *Lentinula edodes* (Shiitake) is the most popular edible mushroom in the world and it has antitumor, immune-modulating, cholesterol-reducing and blood pressure-lowering activities [4-6]. *Lactarius deliciosus* is a well-known mushroom in Turkey and has antioxidant activity [7]. *Lactarius deterrimus* is an ectomycorrhizal species grows on spruce, and found in coniferous forests [8]. The antioxidant capacity of *Lactarius deterrimus* was reported [1]. *Terfezia boudieri* grows in mostly countries around the Mediterranean region, North Africa and Middle East. Similar to other mushroom species in this study, *Terfezia boudieri* has antimicrobial and antioxidant activities [2]. As mentioned above, a lot of benefits of mushrooms show that the need to investigate the new biological activities of them.

Carbonic anhydrase (CA, EC 4.2.2.1) is a zinc containing metalloenzyme and catalyzes the reversible hydration of carbon dioxide to bicarbonate and proton [9]. This reaction is simple but important for the transport of CO<sub>2</sub> and bicarbonate between metabolizing tissues and lungs, pH regulation, electrolyte secretion and some biosynthetic reactions [10]. Because of the critical mission of this reaction, also carbonic anhydrase isozymes having critical importance in the body. So inhibition of CA isozymes is important for treatment of some disorders such as glaucoma, obesity, cancer and osteoporosis [11]. Also activation of these isozymes is important pharmacologically for treatment of Alzheimer's disease [12]. Sixteen different mammalian CA isozymes are described so far. Among the mammalian CAs, hCA I and hCA II are present in humans [13]. Also hCA II is took office in the production of aqueous humor [14]. Regulation of the activity of hCA II in ciliary process has an important place in the treatment of glaucoma. Today some synthetic hCA II inhibitors are used in treatment of glaucoma. But they have some side effects such as weight loss, blurred vision and burning of the eye [15]. Developing

inhibitors have minimized side effects would be an important step in the treatment of this disease. In this context, natural compounds and extracts are of great importance.

In this study, the effects of natural compounds, edible mushroom extracts, on hCA I and hCA II isozymes have been investigated. In addition, the effect of natural compounds in the prevention of disorders related to metabolic regulation of carbonic anhydrase isozymes have been discussed.

## MATERIALS AND METHODS

### Materials

The mushroom species used in this study were collected in Kütahya, Turkey. All chemicals used were analytical grade and were commercially purchased from Sigma-Aldrich Chem. Co. Separation of lysed and non lysed erythrocytes were performed by using centrifuge (SIGMA 3K30, Osterode am Harz, Germany). In the affinity chromatography step peristaltic pump (ISMATEC REGLO Digital MS-2/6, Wertheim, Germany) was used. Esterase activities of the isozymes were measured with UV-Vis spectrophotometer (SHIMADZU UV-1700 PharmaSpec, Kyoto, Japan). Purification of hCA I and hCA II from human erythrocytes.

Erythrocytes were purified from human blood. The blood samples were centrifuged at 1500 rpm for 20 min and plasma was removed. Later, red cells were washed with isotonic solution (0.9% NaCl), and the erythrocytes were hemolyzed with 1.5 volumes of ice-cold water. Cell membranes were removed by centrifugation at 4°C, 20000 rpm for 30 min. The pH of hemolysate was adjusted to 8.7 with solid TRIS (tris(hydroxymethyl)aminomethane). The hemolysate was applied to affinity column (Sephacrose®4B-L-tyrosine-*p*-aminobenzene sulfonamide) pre-equilibrated with 25.0 mM TRIS-HCl/0.1M Na<sub>2</sub>SO<sub>4</sub> (pH 8.7). After extensive washing with a solution of 25.0 mM TRIS-HCl/22.0 mM Na<sub>2</sub>SO<sub>4</sub> (pH 8.7), the hCA I and hCA II isoenzymes were eluted with the solution of 1.0 M NaCl/25.0 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 6.3) and 0.1 M NaCH<sub>3</sub>COO/0.5 M NaClO<sub>4</sub> (pH 5.6), respectively [16]. For quantitative protein determination, the

Bradford method was used with bovine serum albumin as a standard [17]. Also purity control of the isoenzymes was performed with SDS-PAGE after the purification [18].

#### Determination of hydratase and esterase activities of hCA I and hCA II

The CO<sub>2</sub> hydratase activity of the enzyme was determined at 0°C in a veronal buffer (pH 8.15) with the pH-stat method as indicator and saturated carbon dioxide solution as substrate in a final volume of 4.2 mL. The time (in seconds) taken for the solution to change from pH 8.15 to pH 6.50 was measured. The enzyme unit (EU) is the enzyme amount that reduces the non-enzymatic reaction time by 50%. The activity of an enzyme unit was calculated by using the equation  $[(t_0 - t_c)/t_c]$  where  $t_0$  and  $t_c$  are times for pH change of the non-enzymatic and enzymatic reactions, respectively [19].

Esterase activity was assayed by following the change in the absorbance at 348 nm of 4-nitrophenylacetate to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer according to the method described in the literature [20]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M TRIS-SO<sub>4</sub> buffer (pH 7.4), 1.0 mL of 3.0 mM 4-nitrophenylacetate, 0.5 mL H<sub>2</sub>O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

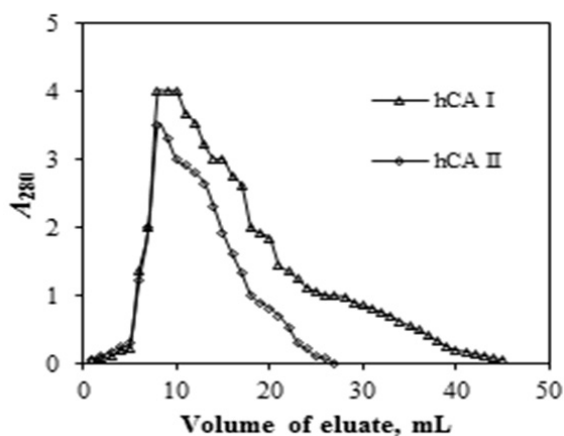
#### Determination of the effects of mushroom extracts on the hydratase and esterase activities of hCA I and hCA II

The stock extracts of *Lentinula edodes*, *Lactarius deliciosus*, *Lactarius deterrimus*, and *Terfezia boudieri* were prepared at 1.0 g/10.0 mL concentrations in ice-cold water. Firstly collected mushrooms were cleaned and were divided into two gram portions. Each piece was individually packaged and frozen at -80°C. Frozen mushrooms were homogenized in 20.0 mL water. To remove insoluble fractions, homogenates were centrifuged at 20000 rpm for 20 min. And then the hydratase and esterase activities of CA isozymes were assayed in the presence of various extract concentrations as mentioned above. The

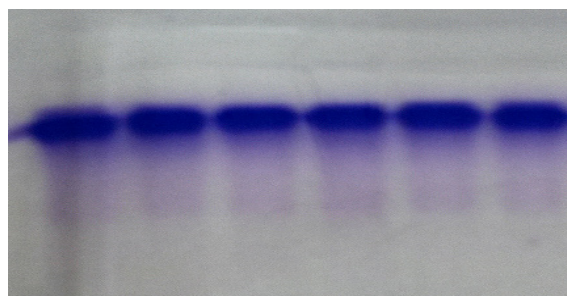
graphics, percent enzyme activity versus extract concentration (mg/mL), were drawn by using Microsoft Excel package program (Excel 2013) and the effects of extracts were determined.

## RESULTS AND DISCUSSION

For investigate the effects of *Lentinula edodes*, *Lactarius deliciosus*, *Lactarius deterrimus*, and *Terfezia boudieri* extracts on human carbonic anhydrase isoenzymes, firstly, hCA I and hCA II isozymes were purified from human erythrocytes by using Sepharose®4B-L-tyrosine-*p*-aminobenzene sulfonamide affinity chromatography. After purification, qualitative protein assay was performed at 280 nm (Figure 1).



**Figure 1.** Qualitative protein assay for purified hCA I and hCA II.



**Figure 2.** SDS-PAGE analysis of hCA I and hCA II (from left to right, first spot is standard hCA I, second and third spots are purified hCA I, and fourth spot is standard hCA II, fifth and sixth spots are purified hCA II).

hCA I was purified 121.56-fold with a specific activity, 1535.31 EU.mg protein<sup>-1</sup> and hCA II was purified 285.19-fold with a specific activity, 3601.90 EU.mg protein<sup>-1</sup> (Table 1). And then purity of the isozymes was checked with SDS-PAGE.

**Table 1.** Summary of purification procedure for hCA I and hCA II.

Purification Step	Activity (EU/mL)	Total Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg protein)	Yield (%)	Purification Factor
Hemolysate	140 20	50.00	11.10	555.00	7010.00	12.63	100.00	1.00
Affinity Chromotography (hCA I)	491 30	5.00	0.32	1.60	2456.50	1535.31	35.04	121.56
Affinity Chromotography (hCA II)	756 40	5.00	0.21	1.05	3782.00	3601.90	53.95	285.19

**Table 2.** The effects of mushroom extracts on the hydratase and esterase activities of hCA I and hCA II.

Extract concentration (mg/ml)	<i>L. edodes</i>		<i>L. deliciosus</i>		<i>L. deterrimus</i>		<i>T. boudieri</i>	
	Activity (%)		Activity (%)		Activity (%)		Activity (%)	
	hCA I	hCA II	hCA I	hCA II	hCA I	hCA II	hCA I	hCA II
0.0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2.0	90.27	92.18	105.72	108.20	105.20	121.14	102.32	112.70
4.0	79.52	78.99	114.11	114.00	109.11	139.06	109.30	169.56
6.0	63.14	69.01	118.02	127.34	115.21	156.03	115.12	195.65
8.0	50.89	57.23	121.01	135.45	124.32	164.47	118.60	229.56
10.0	45.67	48.36	132.12	146.21	130.10	180.25	124.56	248.14
15.0	41.24	44.21	135.45	154.45	136.45	185.89	129.48	273.21
20.0	36.74	36.12	141.24	161.32	140.09	191.02	132.41	299.88

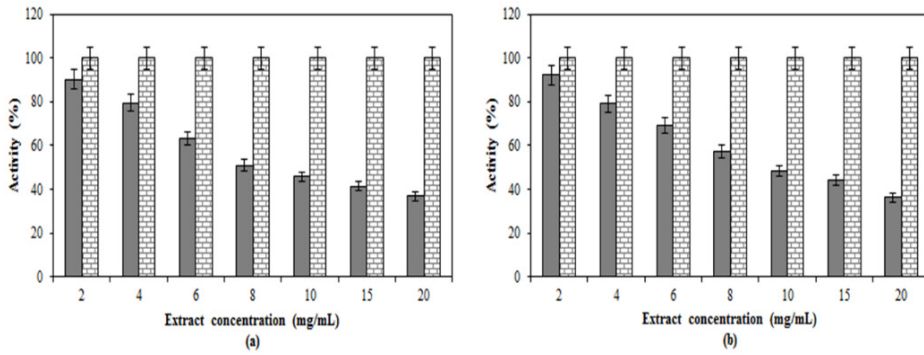
SDS-PAGE spots show that the isozymes have been obtained with high purity (Figure 2).

After purification step, the effect of mushroom extracts on the hydratase and esterase activities of hCA I and hCA II have been investigated. Hydratase activities of the isozymes were not affected by mushroom extracts and have remained constant (Figures 3 and 4). But these extracts were affected the esterase activities of hCA I and hCA II. While *Lentinula edodes* extract has inhibitory effect on hCA I and II, other extracts

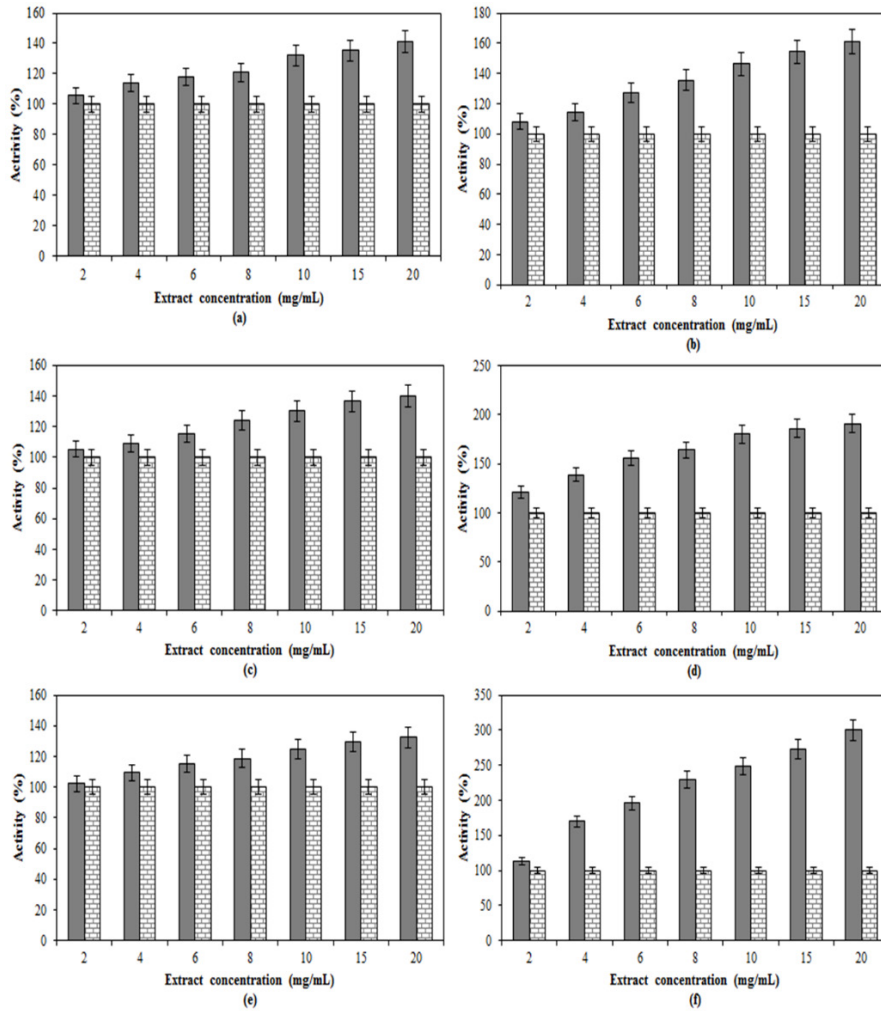
have activator effects on the isozymes.

According to kinetic results *Lentinula edodes* extract have nearly 64.0% inhibition potential on the esterase activities of the isozymes (Table 2, Figure 3). hCA I and hCA II inhibition effect of this extract are close to each other. This situation shows the inhibitor or inhibitors in the extract are not selective inhibitors against an isozyme.

The inhibition of ciliary process enzyme, hCA II, by some agents is important for glaucoma



**Figure 3.** Effect of *Lentinula edodes* extract on hydratase and esterase activities of hCA I and hCA II (a, hCA I and b, hCA II; esterase activity, hydratase activity).



**Figure 4.** Effects of *Lactarius deliciosus*, *Lactarius deterrimus*, and *Terfezia boudieri* extracts on hydratase and esterase activities of hCA I and hCA II. (a, *L. deliciosus* hCA I, b, *L. deliciosus* hCA II; c, *L. deterrimus* hCA I, d, *L. deterrimus* hCA II; e, *T. boudieri* hCA I, f, *T. boudieri* hCA II; esterase activity, hydratase activity).

treatment. Furthermore inhibition of other carbonic anhydrase isozymes are important for the treatment of kidney disorders, obesity, cancer and osteoporosis. This situation, reveals that the existence of *Lentinula edodes* inside eating habits may protect against glaucoma and other risks.

On the other hand *Lactarius deliciosus*, *Lactarius deterrimus*, and *Terfezia boudieri* extracts have activator effects on the isozymes, hCA I and hCA II. Also, the activation effects of these extracts are selective against isozymes. The esterase activation of hCA II are higher than the activation of hCA I. Especially *Terfezia boudieri* has remarkable activation effect (199.88%) on the esterase activity of hCA II (Table 2, Figure 4). The activation potentials of extracts were in the order of *Lactarius deliciosus*  $\approx$  *Lactarius deterrimus* > *Terfezia boudieri* for hCA I, and *Terfezia boudieri* > *Lactarius deterrimus* > *Lactarius deliciosus* for hCA II. In the last decade, it has been explained that carbonic anhydrase activators have an important role in treatment of Alzheimer's disease [12]. Hence consumption of the mushrooms, *Lactarius deliciosus*, *Lactarius deterrimus*, and *Terfezia boudieri*, is likely to help in the prevention of Alzheimer's disease.

In summary, new biological properties of *Lentinula edodes*, *Lactarius deliciosus*, *Lactarius deterrimus*, and *Terfezia boudieri* mushrooms were identified with this study. On the basis of the results it is suggested that the extract of mushroom species evaluated here could be use an accessible source of natural protective agents against some disorders for the nourishment. However, at present, the active components in extracts responsible for the inhibition or activation of hCA I and hCA II isozymes are unknown. So, further studies could be done for determination and purification of the active components from these mushroom extracts. This paper reports the first study performed to investigate the effects of these species on carbonic anhydrase isozymes.

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