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RESEARCH ARTICLE

Investigation of the inhibitory properties of some phenolic standards and bee products against human carbonic anhydrase I and II

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Abstract

Polyphenols are important secondary products of plants with the potential to inhibit carbonic anhydrases. The aim of this study was to investigate the inhibition effects of various phenolic standards, honey, propolis, and pollen species on human carbonic anhydrase I and II. The inhibition values (IC_{50}) of the phenolics (gallic acid, protocatechuic acid, quercetin, catechin, tannic acid, and chrysin) ranged from 0.009 to 0.32 μg/mL, tannic acid emerging as the best inhibitor. The inhibition values of three different types of honey, heather, rhododendron, and chestnut ranged between 2.32 and 25.10 μg/mL, the chestnut honeys exhibiting the best inhibition. The ethanolic extracts of pollen and propolis exhibited good inhibitory properties, with IC_{50} values between 0.486 and 3.320 μg/mL. In order to evaluate the phenolic composition of bee products, phenolic profiles and total phenolic contents (TFC) were also measured. The inhibition ranking among the natural products studied was phenolic standards > propolis > pollen > honeys, and inhibition was related to TFC.

Keywords

Bee product, carbonic anhydrases, inhibition, phenolic

History

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Introduction

Carbonic anhydrases (CA, carbonate hydroliyase, E.C.4.2.1.1) are one of the most studied enzymes. They are present in all species and constitute a family involved in regulating pH and water, electrolyte, and ion transport. Zinc metalloenzymes play an important role in respiration, in the transport of carbon dioxide and bicarbonate, in the pH and CO_2 balance in the lungs and tissues, in the release of secretions from various tissues and organs, and especially in biosynthetic metabolic processes such as lipogenesis, glycogenesis, and ureagenesis in mammals. Carbonic anhydrases are also necessary for CO_2 fixation in plants, algae, and prokaryotes[1,2]. The carbonic anhydrase enzyme is common in organisms but has different isoenzymes that vary according to environmental conditions and requirements. There are currently 16 known isozymes[3]. The most widely available and most studied of these are CA I and CA II. The enzyme exhibits esterase activity in addition to hydrate activity. However, hydrate activity is more important from a physiological standpoint. The enzyme thus plays an important role in the regulation of organisms' acid–base balance. Intervention in carbonic anhydrase enzyme activity is a treatment modality widely applied in cases where this balance is disrupted, such as increased intraocular pressure. From this perspective, carbonic anhydrase inhibitors are clinically highly important compounds[4].

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Acetazolamide, methazolamide, sulfanilamide, dichlorphenamide, and dorzolamide derivatives have been used for many years to reduce intraocular pressure in glaucoma[2,5]. Systemic carbonic anhydrase inhibitors are one of the most powerful agents used to lower intraocular pressure. However, their use is generally accompanied by many undesirable side-effects. These side-effects of drugs used as CA II inhibitors in glaucoma have resulted in a search for natural inhibitors.

Plant-derived natural products have increased in popularity in recent years. Polyphenols are important secondary metabolites of plants with numerous biological active properties, such as antioxidant, antimicrobial, anticarcinogenic, and antiinflammatory effects[6–8]. Plants also have an unlimited ability to synthesize aromatic compounds, as well as phenolics[9]. Honey, propolis, and pollen are important plant-derived bee products containing numerous kinds of phenolic substances[10]. The composition of bee products is reported to vary depending on climatic conditions, flora, environmental conditions, and the state of the colony, and the quality of honey depends on its chemical composition and floral origin[11,12]. There is known to be a positive relationship between the biological active properties and phenolic content of bee products, such as honey, pollen, and propolis[13,14].

The purpose of this study was therefore to investigate various pure phenolic compounds and extracts of bee products from Turkey, including unifloral honeys, pollen, and propolis, in terms of the human CA I and II (hCA I and hCA II) isoforms involved in crucial physiological and pathological processes.

Materials and methods

Reagents

Analytical grade solvents (methanol and ethanol) were obtained from Merck Co. (Merck, Darmstadt, Germany). Buffer and other reagents were the highest purity grade and were obtained from Sigma-Aldrich (Milan, Italy). Carbonic anhydrase isozymes were isolated from human blood cell using affinity chromatography. All standard phenolic compounds, acetazolamide, and sulfanilamide were obtained from Sigma-Aldrich (Milan, Italy). Human CAI and CAII were supplied by Sigma-Aldrich (Germany).

Samples and extractions

Unifloral honeys, pollen, propolis were obtained from experienced bee-keepers in Turkey in 2014. Heather honey I and II samples were obtained from the Mugla region, pollen I samples from the Anzer region, pollen II from Erzurum, propolis I from Ankara, and propolis II from Giresun. Both of the rhododendron honey samples were obtained from Trabzon, and the chestnut honeys were obtained from Sinop and Rize. Unifloral properties of the honeys were tested using melissopalynological characterization. The levels of the major botanical pollen components were between 52% and 84%, since a dominant pollen content more of 45% of total pollen is regarded as constituting unifloral honey[13,15]. The crude samples were stored in a refrigerator at +4 °C. The honey samples were diluted in bidistilled water and then filtered before use. Once the raw propolis samples had been powdered, ethanolic extraction was performed. Approximately 5 g power propolis was extracted with 70% ethanol. Following stirring for 24 h at room temperatures, ultrasonic dissolving was performed (Heidolph Promax 2020, Schwabach, Germany) for 30 min. The suspension was then filtered and centrifuged at 10 000 g for 15 min. Finally, the supernatant was evaporated and lyophilized. The residue was resolved in 70% alcohol. Pollen samples were extracted with 70% ethanol. Following stirring for 24 h, ultrasonic dissolving was performed, as with the propolis samples. All phenolic acid and flavonoid standards were dissolved in 70% ethanol. Acetazolamide and sulfanilamide were dissolved in bidistilled water.

In order to prepare the samples for HPLC analysis, once the extracts had been filtrated, pH was adjusted to 1.0 with HCl. Solid phase extraction was then performed using Supelclean TM LC-18 SPE tubes (Bellefonte, PA) to separate phenolic compounds. The solvents of the ethanolic fractions were evaporated to dryness under reduced pressure in a rotary evaporator at 40 °C. The residue was finally dissolved in ethanol for HPLC analysis[16].

Determination of total phenolic content

Total polyphenol content was estimated following the Folin–Ciocalteu method using gallic acid as reference standard[17]. For analysis, ethanolic extracts of the samples were prepared, and the amount of total phenolic compound was determined based on the gallic acid standard. The absorbance values read at 760 nm were placed on the y-axis, and the concentration values were placed on the x-axis to prepare a standard work graph. The results were calculated as mg gallic acid equivalent(GAE)/100 g sample using the standard work graph prepared.

Determination of phenolic profile

Hitachi HPLC (Elite LaChrom, Hitachi, Japonya) analysis of phenolic compounds was performed on a reverse phase Zorbax Eclipse XDB-C18 column (4.6 X 150 mm, 5 µm particle size), using a gradient program with two solvent systems (A, 0.5% acetic acid in acetonitrile: water (1:1); B, 2% acetic acid in water at a constant solvent flow rate of 1.2 mL/min). An aliquot of 10 µL of sample was injected, and signal detection was carried out at 280 nm and 315 by ultraviolet detection[13].

CA esterase activity and inhibition

The carbonic anhydrase enzyme hydrolyzes the p-nitrophenyl acetate substrate to p-nitrophenol or p-nitrophenolate. The activity is determined by the decrease in absorbance at 348 nm[18,19]. The p-nitro phenyl acetate (p-NFA) used as the substrate in determination was prepared fresh on a daily basis. The enzyme-free part was used as a control during activity measurement. During pipetting, a mixture solution of 0.470 µL 0.05 M, pH 7.4 Tris-SO_4 buffer, 3 mM 0.350 µL of p-NPA, and 180 µL of pure water was used as the control. Instead of reducing the amount of water in the control sample, a control sample was prepared using 50 µL of enzyme solution. Absorbances at minutes 0 and 3 were read at 348 nm, and the difference between them was calculated. The difference between the control value obtained with or without enzyme was calculated, and this value was regarded as corresponding to 100% activity. This differential value needs to be higher than the value measured with the addition of the inhibitor. The inhibitions were expressed as IC_{50} values, representing the concentration of compound producing 50% inhibition of the enzymes. The IC_{50} value was calculated for each inhibitor by taking an inhibitor at a specific concentration, diluting it with five different concentrations, and then drawing a concentration versus a percentage activity graph.

Statistical analysis

The results are presented as mean values plus standard deviations of triplicate measurements. Data were tested using analysis of variance (ANOVA) on SPSS software (version 9.0 for Windows 98, SPSS Inc., Chicago, Il).

Results and discussion

This study evaluated the inhibition effects of various pure phenolic standards and bee products on hCA-I and hCA-II isoenzymes. First, the total phenolic contents (TPC) and phenolic profiles of the bee products were determined. The results are given in Tables 1 and 2. Three different unifloral honey species of heather, rhododendron, and chestnut honeys were used. TPC values for the honeys ranged between 32 and 105 mgGAE/100 g samples, with chestnut honeys containing the highest level

Table 1. RP-HPLC-UV validation parameters of the studied phenolic compounds.

Compounds	R^2	%RSD (retention time)	%RSD (area)	LOD[a]	LOQ[a]
Gallic acid	0.999	0.210	1.941	0.022	0.067
Protocatechuic acid	0.999	0.871	1.920	0.042	0.128
p-OH Benzoic acid	0.998	0.351	3.055	0.036	0.109
Catechin	0.997	0.492	4.279	0.040	0.121
Vanillic acid	1.000	0.828	2.066	0.025	0.075
Caffeic acid	0.998	0.179	4.039	0.062	0.187
Syringic acid	1.000	0.550	0.848	0.009	0.027
Epicatechin	0.999	0.429	3.819	0.030	0.090
p-Coumaric acid	0.999	0.204	1.562	0.010	0.030
Ferulic acid	0.999	0.222	1.301	0.011	0.033
Rutin	1.000	0.234	3.139	0.041	0.123
Daidzein	0.998	0.174	1.545	0.018	0.054
t-Cinnamic acid	1.000	0.262	1.071	0.014	0.042
Caffeic acid phenyl ester CAPE	0.994	0.229	5.833	0.043	0.130

[a]Values are expressed as mg/L.

Table 2. Phenolic composition of the studied honey-bee products (μg/g).

Bee products	GA	ProCA	BA	CLA	VA	SA	CA	COU	FER	CAPE	QUE	CAT	RUT
Heather honey-I	0.521	22.456	28.410	nd	2.490	nd	0.890	3.124	nd	nd	8.560	16.04	4.403
Heather honey-II	0.440	18.876	16.400	nd	1.830	nd	1.402	2.200	nd	nd	12.40	13.76	6.132
Rhododendron honey-I	0.052	1.052	5.780	nd	0.423	nd	4.120	3.980	0.526	nd	2.400	nd	0.090
Rhododendron honey-II	0.034	3.220	11.200	nd	-	nd	0.582	5.407	2.504	nd	3.880	nd	0.055
Chestnut honey-I	1.100	6.560	22.405	nd	2.420	nd	6.450	8.405	2.050	nd	4.220	nd	3.445
Chestnut honey-II	0.880	8.400	14.560	nd	3.050	nd	4.780	5.080	1.470	nd	2.920	nd	1.420
Propolis-I	5.560	7.405	34.150	14.415	28.140	4.400	130.100	72.400	248.025	1478.12	450.730	14.430	102.600
Propolis-II	1.422	3.484	55.330	10.170	34.770	-	241.333	58.203	174.320	898.080	560.450	3.435	124.900
Pollen-I	3.450	nd	8.440	nd	8.450	3.552	24.320	33.550	6.740	146.441	42.455	nd	24.478
Pollen-II	2.056	nd	14.700	1.100	5.510	5.860	16.100	27.110	11.700	210.036	60.036	1.170	30.170

GA: gallic acid; ProCA: protocatechuic acid; BA: *p*-OH benzoic acid; CLA: chlorogenic acid; VA: vanillic acid; SA: syringic acid; CA: caffeic acid; COU: *p*-coumaric acid; CAPE: caffeic acid phenyl ester; QUE: quercetin; CAT: catechin; RUT: rutin.

Table 3. Inhibitory effects of some bee products and polyphenols against hCA I and hCA II.

	Samples	Total phenolic content (mg GAE/100g)	hCA I IC_{50} (μg/mL)	hCA II IC_{50} (μg/mL)
Honey	Heather honey-I	46.770 ± 1.880	8.105 ± 1.105	16.830 ± 1.050
	Heather honey-II	43.270 ± 2.160	7.050 ± 0.082	25.120 ± 1.542
	Rhododendron honey I	32.120 ± 3.120	3.012 ± 0.042	9.720 ± 1.220
	Rhododendron honey II	24.151 ± 1.062	3.580 ± 0.072	10.450 ± 0.556
	Chestnut honey I	105.206 ± 2.550	1.320 ± 0.152	2.323 ± 0.024
	Chestnut honey II	94.404 ± 1.682	1.522 ± 0.222	2.880 ± 0.036
Propolis	Propolis-I	850.460 ± 12.400	1.273 ± 0.352	0.486 ± 0.032
	Propolis-II	788.120 ± 10.520	1.374 ± 0.066	0.612 ± 0.010
Pollen	Pollen-I	255.20 ± 12.330	1.410 ± 0.032	3.320 ± 0.028
	Pollen-II	247.620 ± 5.250	1.144 ± 0.010	2.880 ± 0.020
Polyphenols	Gallic acid	–	0.009 ± 0.000	0.035 ± 0.012
	Protocatechuic acid	–	0.134 ± 0.005	0.320 ± 0.020
	Quercetin	–	0.008 ± 0.001	0.019 ± 0.002
	Catechin	–	0.002 ± 0.000	0.034 ± 0.002
	Tannic acid	–	0.035 ± 0.000	0.009 ± 0.001
	Chrysin	–	0.017 ± 0.001	0.044 ± 0.001
CA inhibitors	Acetazolamide	–	0.011 ± 0.002	0.004 ± 0.001
	Sulfanilamide	–	0.014 ± 0.002	0.014 ± 0.001

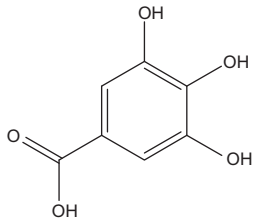
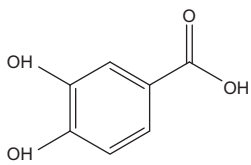
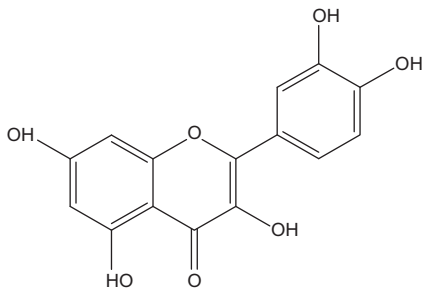
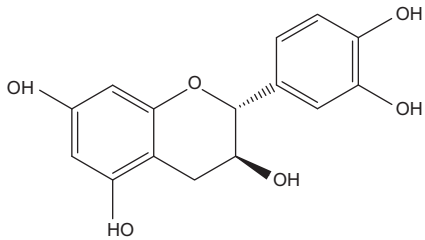
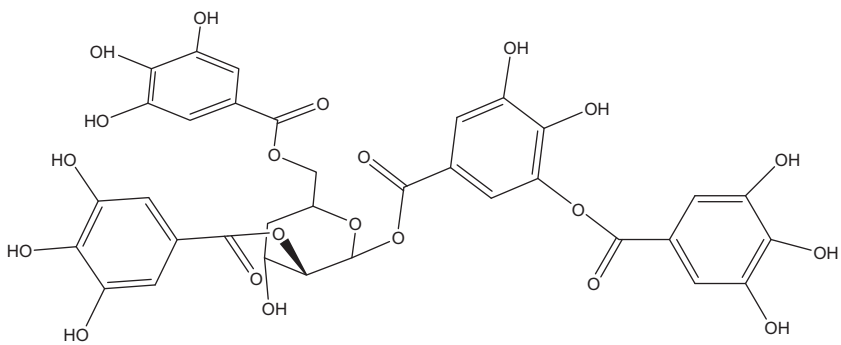
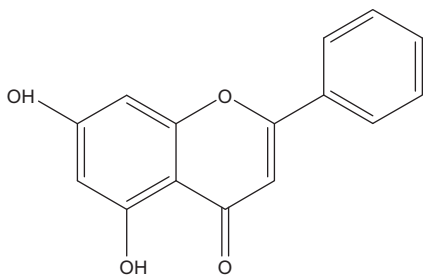
of TPC. In agreement with this finding, one comparative study of unifloral honeys produced in Turkey also reported that chestnut honeys were contained the highest level of TPC[13,20,21]. Chestnut honey is a dark-blossom honey reported to contain higher antioxidant and antimicrobial properties than light-color blossom honeys[13,14]. Total phenolic contents of the propolis samples ranged between 788 and 850 mg GAE/100 g, values nearly 10-fold higher than those of the honeys. Propolis is not obtained in pure form from a single source, and the collection areas are rich in chestnut, poplar, hazelnuts, and cherry trees. TPC values of the pollen samples ranged between 247 and 255 mg/100 g, lower than the propolis samples, but higher than the honeys. Ranking the bee products according to their TPC levels gave the order propolis > pollen > honey.

In order to determine phenolic compositions of the honeys, pollens, and propolis, 13 phenolic standards were analyzed qualitatively and quantitatively using RP-UV-HPLC. The results are given in Table 1. All phenolic compounds were detected in different varieties and quantities in the bee product samples. Propolis samples contained the four highest phenolic substances, with quercetin, rutin, CAPE, ferulic acid, and coumaric acid being identified as the major components of the samples. Various previous propolis studies have reported CAPE, quercetin, and rutin as the major phenolic components[22–24]. Benzoic acid, coumaric acid, and catechin were determined at higher levels in the chestnut honeys than the other honeys. Our findings are compatible with those of previous studies[13,20,25]. Heather honey is a dark honey, and both the heather honeys in this study were rich in flavonoids of quercetin, catechin, and rutin[13,26]. The lowest TPC and phenolic composition in this study were observed in the rhododendron honeys, light-colored blossom honeys[13,14]. Rhododendron honey is also known as 'mad honey' since it contains the toxic compounds andromedotoxin, acetylandromedol, rhodotoxin species, polyhydroxylated cyclic diterpenes, and grayanotoxin, a diterpene and polyhydroxylated cyclic hydrocarbon that does not contain nitrogen[21,28].

Natural compounds and natural extracts exhibit many biological activities, such as inhibition and activation of some clinically important enzymes, such as hCA, urease, acetylcholine esterase, xanthine oxidase, monoamine oxidase, and hyaluronidase[10,27,29–31]. In this study, we measured the inhibition effect on hCAI and hCAII of various bee products and phenolic standards and compared the results with acetazolamide and sulfonamide inhibitors (Table 3). In additional to having positive effects on glaucoma, CAs inhibitors are reported to exhibit numerous other potential activities, such as anti-cancer, anti-obesity, and anti-infective properties and are used in the treatment of Alzheimer's disease[32–34].

Of the six different phenolic compounds in the present study (Table 4), catechin was identified as the best potential inhibitor of

Table 4. Phenolic standards found in the studied samples.

Phenolic standards	Molecules formulas
Gallic acid	
Protocatechuic acid	
Quercetin	
Catechin	
Tannic acid	
Chrysin	

hCAI, and tannic acid as the best inhibitor of hCAII. The ranking of the inhibitory effects on hCAI of the phenolic standards was in the order catechin > quercetin = gallic acid > chrysin > tannic acid > protocatechuic acid, while for hCAII the ranking was tannic acid > quercetin > gallic acid = chrysin = catechin = protocatechuic acid. No correlation was determined between either enzyme in terms of phenolic inhibitions. Although tannic acid exhibited the highest inhibition of hCAII, catechin was the most effective against hCAII. A naturally occurring plant polyphenol, tannic acid is composed of a central glucose molecule derivatized at its hydroxyl groups with one or more galloyl residues, and exhibits good antioxidant activity[34]. Apart from the phenolic compounds investigated in this study, a wide series of polyphenolic, including resveratrol, dobutamine, curcumin, catechin, and silymarin, have been investigated in terms of the inhibition of all the catalytically active mammalian isozymes of carbonic anhydrase, and these have been reported to exhibit different inhibition effects on the enzymes[2]. Catechin has been observed to exhibit effective inhibition of all 13 investigated CA isoforms, including hCA-VII, hCA-II, and hCA-I.

This study investigated the inhibition properties of three different unifloral honeys, heather, rhododendron, and chestnut. Chestnut honey exhibited the highest level of inhibition of both hCA isoforms. The honey inhibition values (IC_{50}) were statistically divided into three categories. The inhibition ranking was chestnut > heather > rhododendron, and inhibition was also correlated with honeys' TPC.

Of the three different bee products investigated, propolis exhibited the best inhibition concentrations, with values ranging from 0.480 to 1.374 μg/mL. It also exhibited higher hCA-II inhibition. The pollen samples also exhibited inhibition of both hCAs. In contrast to propolis, the inhibition concentrations were also effective against the hCAs. In agreement with our results, other studies have also reported that propolis exhibits the highest inhibition of hCAs among propolis, pollen, and honey.

In conclusion, we investigated the potential inhibition of hCA-I and hCA-II using a group of polyphenolic derivatives and bee products. All the polyphenols effectively inhibited these enzymes, with IC_{50} values in the range of 0.002 μg/mL–0.350 μg/mL. Tannic acid exhibited good inhibition potential against hCA-II and catechin against hCA-I. We think that these data may assist with the design of more effective forms of hCA-I and hCA-II.

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Declaration of interest

The authors declare no conflict of interest with the data from this paper.

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