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**SHORT COMMUNICATION**

# Honey, polen, and propolis extracts show potent inhibitory activity against the zinc metalloenzyme carbonic anhydrase

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**Abstract**

Three different honey extracts from the endemic plant in the Black Sea region *Rhododendron ponticum*, were investigated for their inhibitory effects against the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), more precisely the human (h) isoforms hCA I and hCA II. Hexane, methanol, ethanol, and water solid-phase extractions (SPEs) showed inhibitory activity towards the two CA isozymes which were related to the total phenolic content. The highest inhibitory effects (0.036–0.039 mg/mL) were those of propolis methanolic extract. Among the three different samples investigated here, the aqueous extracts showed lower inhibitory effects compred to the organic solvent SPE extracts (in the range of 1.150– 5.144 mg/ mL). The studied honey extracts constitute an interesting source of phenolic derivatives that might serve to identify lead compounds, targeting the physiologically relevant enzymes CA I and CA II.

**Keywords:** Carbonic anhydrase, honey, *Rhododendron ponticum*, natural polyphenols, isoform I and II.



## Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc enzymes, present in prokaryotes and eukariotes, being encoded by five distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, *Bacteria*, algae, and cytoplasm of green plants), the β-CAs (predominantly found in *Bacteria*, algae and chloroplasts of both mono- as well as dicotyledons), the γ-CAs (mainly present in *Archaea* and some *Bacteria*), and the δ- and ζ-CAs, present in marine diatoms, respectively[1–8]. In mammals, 16 different α-CA isozymes or CA-related proteins were described, with very different subcellular localization and tissue distribution. Basically, there are several cytosolic forms (CA I–III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII, and CA XIV), one mitochondrial form (CA V), as well as a secreted CA isozyme, CA VI[1–8]. These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of $CO_2$/bicarbonate between metabolizing tissues and lungs, pH and $CO_2$ homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic, or pathologic processes[1–8]. Many of these mammalian (human) isozymes are important targets for the design of inhibitors with clinical applications[1].

Several classes of CA inhibitors (CAIs) were describe so far, such as the metal-complexing anions, the unsubstituted sulfonamides and their bioisosteres (sulfamates, sulfamides), the phenols and thiophenols, which generally bind to the Zn(II) ion of the enzyme either by substituting the non-protein zinc ligand to generate a tetrahedral adduct[1–3] or by addition to the metal coordination sphere[1], generating trigonal-bipyramidal species. Phenols bind to the zinc-coordinated water molecule/hydroxide ion. At least 25 clinically used drugs (sulfonamides and sulfamates) have been reported to possess

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significant CA inhibitory properties, and they have applications as diuretics and anti-glaucoma drugs, but it has recently emerged that CAIs could have potential as novel anti-obesity, anti-cancer, and anti-infective drugs[1]. Furthermore, recent studies suggest that CA activation may provide a novel therapy for Alzheimer's disease[1].

Recently, we have described several natural products based CAIs, which evidenced diverse chemotypes than the sulfonamides and their bioisosteres, such as for example natural product phenols[9,10], the coumarins[11,12] and other such compounds. This led to a strong diversification of the chemotypes known to show strong activity as enzyme inhibitors and also to the discovery of a novel mechanism of inhibition of CAs with coumarins and structurally similar derivatives binding to the enzyme in a totally new manner, without interacting with the catalytically crucial metal ion[11–13]. For example, the natural product coumarin **1a** was shown to be hydrolyzed enzymatically by the esterase CA activity to the *cis*-2-hydroxycinnamic acid derivative **1b**, which occludes the entrance to the enzyme active site, and behaves as a low nanomolar CAI[11]. The same is true for the simple coumarin **2a**, but in that case the CAIs is the *trans*-2-hydroxycinnamic acid **2b**[12]. Another interesting CAIs recently identified is the diphenolic fungal metabolite **3**, which showed low nanomolar inhibitory activity against some mitochondrial CA isoforms[9]. Thus, natural products seem to be a valuable source of enzyme inhibitors targeting CAs, enzymes involved in many pathologic states[1].

Honey, pollen, and propolis are highly valuable products of the domestic bee (*Apis mellifera*) The composition of these products is rather variable, and depends on many biogeographical conditions such as the plant types, climate, environmental conditions, and contribution of the beekeeper[14]. These food products have been used in folk medicine since the early ages of human history, whereas more recently their role in the treatment of burns, gastrointestinal disorders, asthma, infected wounds, and skin ulcers has been also reinvestigated[15–17]. These food products have been reported to contain about 150–200 ppm compounds such as polyphenols (phenolic acids, flavonoids, and their derivatives), terpenes, steroids, amino acids, etc.[15,18,19]. Many studies reported that propolis, pollen, and honey contain a variety of phenolic acid and flavonoids, possessing a wide range of biological effects including antioxidant, anti-bacterial, and anti-inflammatory activities[20–22].

Considering our interest in natural products as sources of novel inhibitors of metalloenzymes, and particularly of the CAs, in this study, we used three honeybee samples of rhododendron honey, pollen, and propolis which are known to have high phenolic compounds content for investigation as CAIs[19]. The endemic to the Black Sea rhododendron species *Rhododendron ponticum*, gives a honey which is locally known as "mad" or wild honey, and is being produced only in the Black Sea region[19]. This honey contains grayanotoxins (formerly known as andromedotoxins, acetylandromedol, or rhodotoxins) which are polyhydroxylated cyclic diterpenes possessing structures **4**[19,23]. *Rhododendron ponticum* leaves and flower nectar (including honey made from plant nectar) are sources of these toxins[23]. The symptoms of poisoning due to the consumption of large amounts of this honey include sudden severe hypotension, bradycardia, and vertigoes[23]. There is an anecdotical mentioning of mad honey, from the classic helenistic period, by Xenophon, towards 410 BC (*Anabasis*). In the Black Sea mountains the soldiers hired by Cyrus the younger and led by Xenophon (when returning from their expedition against Artaxerxes II) who consumed mad honey fainted and stayed unconscious until the next day (Xenophon, *Anabasis*).

Propolis is a resinous natural substance which is used to protect hives from bacterial/fungal infections, being collected from plants and tree buds or seep from the tree bark. Ethanolic propolis extracts were used in antioxidant preprations, as throat spray, or as an ingredient in cosmetics and toothpaste, showing anti-bacterial, antiviral, antioxidant, anti-cancer, and anti-inflammatory activities[24,25].

Pollen constitutes the reproductive cells of plants. Bees consume pollen in their own diets and use it to feed larvae. Pollen is used as a dietary supplement for human diet and it is reported to be a source of phenolic compounds with a host of biological activities[22].

The aim of the study was to investigate the *in vitro* inhibitory effects of bee honey products including mad honey from *R. ponticum*, on human CA isoforms involved in crucial physiologic and pathologic processes, such as CA I and CA II. As far as we know, this is the first study investigating the inhibition of CAs with food-derived products.

## Materials and methods

### Reagents

Analytical grade solvents (methanol, ethanol, and hexane) were obtained from Merck Co. (Merck, Darmstadt, Germany). Buffers and other reagents were the highest purity grade, from Sigma-Aldrich (Milan, Italy). CA isozymes were recombinant ones, obtained as reported earlier[5,9,10]. Honey was from *R. ponticum* flowers (mad honey) whereas the pollen and propolis were multiflower ones, commercially available samples from the Trabzon region, Black Sea, Turkey.

### Samples and preparation of extracts

Propolis, pollen, and honey samples were supplied by chairmanship of Zonguldak, Türkiye Honey Agricultural Cooperative as the product of whole season of 2008. The crude samples were stored at +4°C in refrigerator. About 5 g of sample was weighed and added to 100 mL solvent (methanol, ethanol, hexane, and water). Then, each sample was continuously stirred with a shaker at room temperature for 24 h. The suspension was removed by centrifuged at 10,000*g* for 15 min. Then, the supernatant

was concentrated in a rotary evaporator under reduced pressure and the residue resolved in a minimal volume of the same solvent and kept in 4°C until used. In addition honey, pollen, and propolis, 5 g each, were dissolved in 50 mL methanol and the mixture was periodically stirred and the supernatant was filtered twice with Whatman no.4 and filter papers, respectively. The filtrate was applied to a solid-phase extraction (SPE) column (Supelco-LC18, Milan, Italy). Elution of phenolic from the column was performed with methanol and concentrated in a rotary evaporator.

### CA catalytic activity and inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed $CO_2$ hydration activity[26]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffers, and 20 mM $Na_2SO_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed $CO_2$ hydration reaction for a period of 10–100 sec. The $CO_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each sample, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Solutions of extracts (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitory sample and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the enzyme-inhibitor complex. The $IC_{50}$ reprsents the concentration of inhibitor producing a 50% decrease of the catalytic rate and were obtained by non-linear least-squares methods using PRISM 3, and represent the mean from at least three different determinations.

### Determination of total phenolic content

The content of total polyphenols was estimated according to the Folin-Ciocalteu method using gallic acid as reference standard[19]. According to this procedure, the extracted solution was previously diluted in the proportion of 1:10 (0.5 mL) and then mixed with 0.5 mL of 0.2 N Folin–Ciocalteau reagents and 1.5 mL of 2% $Na_2CO_3$. The absorbance was read at 760 nm after 2 h of incubation at room temperature. Total phenolic content was expressed as mg of gallic acid equivalents per g of extract, by using a Standard graph. Among three honey bee's samples, all propolis extracts showed the highest inhibitory effects ranging from 0.05 to 2.25 mg/mL.

### Statistical analyses

Results are presented as mean values of two replicates. Data were tested using SPSS for Windows Release 10 (SPSS Inc. Chicago, IL). Pearson correlation was used to reveal differences and relations. Significant differences were statistically considered at the level of $p < 0.05$ otherwise given.

## Result and discussion

Honey, pollen, and propolis are biologically active food products, probably containing hundreds of diverse natural substances[14,15]. These food products are rich in phenolic compounds[14,19], and are becoming increasingly popular because of their potential role in contributing to human health. The composition of such products is variable and depends on several factors. Many studies reported that they contain a variety of flavonoids and phenolic acids (both benzoic and cinnamic acid derivatives), possessing a wide range of biological effects including anti-bacterial, anti-inflammatory, anti-allergic, and anti-thrombotic activities which make honey and derived products interesting antioxidants[14–20]. As far as we know, such products have been never tested for their potential inhibitory activity against the CA family of enzymes.

Indeed, CAs are physiologically relevant enzymes since they are present in a multitude of isoforms in various tissues, being involved in pH regulation, secretion of electrolytes, diuresis, $CO_2$ transport and metabolism (biosynthetic reactions such as gluconeogenesis, ureagenesis, lipid biosynthesis)[1–7]. Therefore, CA has been a long established target for drug design, with several sulfonamides of the type $RSO_2NH_2$ being used clinically since 1956, when acetazolamide AAZ, the first of these was approved as a diuretic 1]. Sulfonamides are now widely used drugs for the treatment or prevention of a variety of diseases such as glaucoma, gastroduodenal ulcers, acid-base disequilibria and diverse neurological/neuromuscular disorders[1–7,27–29].

We have prepared several different solvent extractions (hexane, methanol, ethanol, water, and methanolic SPE extracts) of mad honey from *R. ponticum*, pollen and propolis and measured their total phenolic contents (Table 1). It should be mentioned that normal multiflower honey does not show any CA inhibitory activity (data not shown). The highest phenolic substance was found in the propolis extracts. Especially, the SPE column extracts showed higher phenolic content than other preparations because of the SPE (C-18) column specific for phenolic compounds separation. It may be observed that in the honey samples the total polyphenol content was between 10.48 and 95.46 mg/g of sample, whereas for the pollen and propolis the amounts were rspectively of 50–235.5 mg/g sample and of 13.45–464.4 mg/g sample (Table 1).

We measured thereafter the inhibitory effects of these samples against purified CA isoforms hCA I and II, which are the physiologically most relevant such enzymes[1,27–29]. The inhibition results are expressed as $IC_{50}$ (mg/mL), Table 1. The meaning of $IC_{50}$ is the extract concentration which reduces the CA activity by 50%. Among all the investigated samples, it may be observed that the extraction method highly influenced the CA inhibitory activity

Table 1. Inhibitory effects of honey, pollen, and propolis extracts against hCA I and hCA II, from three different determinations.

| Samples | Solvent | Concentration of extracted sample | Total polyphenolmg GAE/g sample | hCA I $IC_{50}$ (mg/mL) | hCA II $IC_{50}$ (mg/mL) |
|---|---|---|---|---|---|
| Honey (Mad Honey; produced from *Rhododendron ponticum*) | Hexane | 0.2 g/mL | 10.86 ± 0.03 | 0.123 | 0.125 |
| | Methanol | 0.2 g/mL | 11.97 ± 0.02 | 0.154 | 0.171 |
| | Ethanol | 0.2 g/mL | 10.76 ± 0.01 | 1.705 | 2.830 |
| | Water | 0.2g/mL | 10.48 ± 0.02 | 2.961 | 3.150 |
| | SPE column | 0.01 g/mL | 95.46 ± 0.04 | 0.524 | 0.690 |
| Pollen | Hexane | 0.08 g/mL | 50.06 ± 0.05 | 0.337 | 7.680 |
| | Methanol | 0.08 g/mL | 52.40 ± 0.04 | 1.79 | 9.113 |
| | Ethanol | 0.08 g/mL | 54.27 ± 0.03 | 1.602 | 7.540 |
| | Water | 0.08 g/mL | 56.60 ± 0.03 | 2.72 | 5.144 |
| | SPE column | 0.01 g/mL | 235.56 ± 0.04 | 0.260 | 1.010 |
| Propolis | Hexane | 0.08 g/mL | 349.42 ± 0.10 | 0.056 | 0.066 |
| | Methanol | 0.08 g/mL | 267.69 ± 0.16 | 0.066 | 0.087 |
| | Ethanol | 0.08 g/mL | 257.42 ± 0.21 | 0.079 | 0.081 |
| | Water | 0.08 g/mL | 13.45 ± 0.05 | 2.250 | 1.150 |
| | SPE column | 0.005 g/mL | 464.40 ± 0.50 | 0.036 | 0.039 |
| AAZ (acetzolamide) | — | — | — | 365 nM | 25 nM |

against both isoforms. Thus, for honey, against hCA I the inhibition was in the range of 0.123–2.961 mg/mL, with the best inhibition being observed for the hexane extracts, and the worst one for the water extract. Against hCA II, the $IC_{50}$ were in the range of 0.125–3.150 mg/mL, again with best inhibition being observed for the hexane extract and the worst one for the water extracts (Table 1).

For the pollen extracts, against hCA I, the $IC_{50}$ were in the range of 0.260–2.72 mg/mL, whereas against hCA II they were in the range of 1.01–9.11 mg/mL. In this case the most inhibitory activity against both isoforms was observed for the SPE column extracts, followed by the hexane extract (against hCA I) and the water extract (against hCA II). Propolis showed the highest inhibitory effects against both CA isozymes (Table I). Indeed, against hCA I the $IC_{50}$ were in the range of 0.0368–2.25 mg/mL whereas against hCA II in the range of 0.039–1.15 mg/mL, respectively. The order of the inhibitory effects on hCA I and hCA II for the extracts was as follows: propolis > pollen > rhododendron honey samples (meaning the corresponding various extracts in diverse solvents). In all the samples, we obtained a good negative correlation ($R = -0.58$, $p < 0.05$) between total phenolic compounds and the CA inhibition (Table 2). However, the negative correlation was higher in propolis extracts (R = –0.87 to –0.88, $p < 0.01$) and pollen extracts (R = –0.56 and –0.90, $p < 0.05$) than for honey extracts. Although mad honey was not containing high amounts of phenolic compounds (Table 1), it showed a good inhibitory effects on CA I and CA II. Especially the hexane and methanolic extracts of mad honey exhibited a good inhibitory effect. The Pearson correlation analysis of Table 2 also showed a good correlation between the phenolic compounds content of the investigated samples and the inhibition of hCA I and hCA II isozymes. It is thus probable that the inhibitory effects we report may be indeed due to such biologically active components as the polyphenols[30] or the grayanotoxins.

Table 2. The results of statistical analyses for correlation (R) between phenolic compounds content and inhibition of hCA I and hCA II isozymes.

| | hCA I | hCA II |
|---|---|---|
| Total phenolic compounds | –0.58 | –0.45 |
| In honey extracts phenolic compounds | –0.27 | –0.28 |
| In pollen extracts phenolic compounds | –0.56 | –0.90 |
| In propolis extracts phenolic compounds | –0.87 | –0.88 |

Grayanotoxin species from mad honey samples can show inhibitory effects against the CA I and CA II isozymes, but this hypothesis warrants further studies. The grayanotoxins may inhibited CAs by various mechanisms of action, resembling the interaction of CAs with phenols or coumarins, among others[10–12,27–30]. Work is in progress in our laboratories to elucidate the chemical nature of the compounds responsible for these inhibitory activty of honey and related food products against CAs.

## Declaration of interest

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