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RESEARCH ARTICLE

Honey as an apitherapic product: its inhibitory effect on urease and xanthine oxidase

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Abstract

The aim of this study was to evaluate new natural inhibitor sources for the enzymes urease and xanthine oxidase (XO). Chestnut, oak and polyfloral honey extracts were used to determine inhibition effects of both enzymes. In addition to investigate inhibition, the antioxidant capacities of these honeys were determined using total phenolic content (TPC), ferric reducing antioxidant power (FRAP), and DPPH radical scavenging activity assays. Due to their high phenolic content, chestnut and oak honeys are found to be a powerful source for inhibition of both enzymes. Especially, oak honeys were efficient for urease inhibition with 0.012–0.021 g/mL IC_{50} values, and also chestnut honeys were powerful for XO inhibition with 0.028–0.039 g/mL IC_{50} values. Regular daily consumption of these honeys can prevent gastric ulcers deriving from *Helicobacter pylori* and pathological disorders mediated by reactive oxygen species.

Keywords

Antioxidant, chestnut, oak, urease, xanthine oxidase

History

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Introduction

Urease is an enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia[1]. Levels of this enzyme, which is responsible for reducing urea accumulation, are also involved in the development of urolithiasis, pyelonephritis, hepatic encephalopathy, hepatic coma urolithiasis, and urinary catheter encrustation in humans and animals[2]. Urease activity is essential for buffering the acidic pH value in the stomach, nutrient acquisition and improving the ability of *Helicobacter pylori* to colonize the gastric epithelium[3]. Its inhibition is very important for the treatment of *H. pylori*-related diseases.

In addition to this direct potential disease risk, reactive oxygen species (ROS), which are formed through various pathways, are also significant risk factors for diseases in different systems[4]. ROS are involved in oxidative damage to lipids, proteins, and nucleic acids. In general, ROS are formed through both physiological and non-physiological pathways. Some of the enzymes of myeloperoxidase, aldehyde oxidase, nitric oxide synthase and xanthine oxidase (XO) catalyze the formation of some ROS[5,6]. Many ROS are generated by XO to catalyze the oxidation of hypoxanthine into xanthine[7,8]. XO is responsible for oxidative damage that causes many pathological diseases, such as gout, hyperuricemia, hepatitis, carcinogenesis, and aging[9,10]. The regulation of XO is an important means of preventing inflammation[6]. In particular, increased XO expression can result in significant vascular endothelium damage[11], as well as atherosclerosis. XO inhibitor scanning can prevent the development of endothelial dysfunction and atherosclerosis[11,12]. Some chemicals, such as allopurinol[8] and pyrazoles[13], have been used for only clinically XO inhibitor. Besides these chemicals, a number of natural compounds, such as caffeic acid[11,14,15], rutin[16], and chestnut honey[17], have been reported to inhibit XO, and foods rich in phenolic compounds are recommended for reducing blood concentrations of uric acid in gout.

Honey is one of these natural products, and is rich in phenolics as antioxidant. The antioxidant capacity of honey is affected by several factors, such as the floral source involved, and seasonal, geographical and environmental conditions[18,19].

Nasuti et al.[17] and Can et al.[19] investigated the biological properties of some honeys. They reported dark honeys chestnut and oak possessing higher levels of phenolic compounds and antioxidant capacities, and greater apitherapic functions. Another finding of these studies is that phenolic acids and flavonoids in honey could be used as a vital enzyme inhibitors[17,19]. According to another aspect, the use of natural products, rather than chemical-based drugs, is desirable in the inhibition of these enzymes[3,20,21]. Recent studies have revealed that natural products are effective in the elimination of ROS and as enzyme inhibitors[22–24]. Considering these information, the objective of this study is to determine the antioxidant properties and inhibition effects of different types of honey (oak, chestnut and polyfloral honey) on urease and XO for the first time.

Materials and methods

Reagents

Enzymes and their substrates were supplied in the form of bovine milk XO -xanthine and jack bean urease-urea by Sigma-Aldrich (St. Louis, MO). Hydrochloric acid (HCl), glacial acetic acid and Folin–Ciocalteau reagent were obtained from LiChrosolv® (Merck KGaA, Darmstadt, Germany). High quality ultra-pure water was supplied by Human Zeneer Navi Power I Integrate

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(Human Corporation, Seoul, South Korea). Potassium phosphate dibasic (K_2HPO_4), ethylenediaminetetraacetic acid (EDTA), lithium chloride (LiCl), phenol, sodium nitroprusside, sodium hydroxide (NaOH), sodium hypochlorite (NaOCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$), gallic acid, sodium carbonate (Na_2CO_3), sodium acetate trihydrate ($NaCH_3COO{\cdot}3H_2O$), iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), iron(II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), and DPPH (2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl) were purchased from Sigma-Aldrich (St. Louis, MO). LC syringe filters (RC-membrane, 0.2 μm) were obtained from Sartorius Minisart RC 15, Sartorius (Darmstadt, Germany).

Honey samples

The samples were collected by experienced beekeepers in the 2014 harvest season. Three varieties of honey, chestnut (*Castanea sativa* Mill.), oak (*Quercusrobur* L.), and polyfloral honey were used in this study. For tagging, melissopalynological analysis was performed following the method described by Louveaux et al.[25]. Acetolyzed pollen grains were mounted on glycerin jelly and sealed with paraffin. In order to determine the pollen-type frequency classes, 500 pollen grains were counted and classified in terms of dominant pollen (more than 45%). Pollen analyses and honey properties are given in Table 1.

Honey preparation

Approximately 5 g of honey sample was extracted with 20-mL distilled water in a flask attached to a condenser at 60 °C, over 6 h. The extract was subsequently filtered to remove particles, and the final volume was adjusted with distilled water.

Urease inhibition assay

The urease inhibiting activity of the aquatic honey samples was determined by measuring ammonia production using the indophenol method[26]. Briefly, reaction mixtures including 200-μL jack bean urease, 500 μL of buffer (100 mM urea, 0.01 M K_2HPO_4, 1 mM EDTA and 0.01 M LiCl, pH 8.2) and 100 μL honey extract samples were incubated at room temperature for 20 min. Phenol reagent (550 μL, 1% w/v phenol and 0.005% w/v sodium nitroprusside) and alkali reagent (650 μL, 0.5% w/v sodium hydroxide and 0.1% v/v NaOCl) were added, and the increasing absorbance at 625 nm was measured after 50 min, using a Thermo Scientific Evolution 260 spectrophotometer (Thermo Scientific, Waltham, MA). The IC_{50} value was determined as the concentration of sample causing 50% inhibition of maximal activity.

In vitro anti-xanthine oxidase assay

The XO inhibitory activity of honey extracts was determined using the UV spectroscopy technique at 295 nm[27] with some slight modifications. The reaction mixture consisted of 0.5 mL of the test compound, 0.77 mL of phosphate buffer (pH 7.8) and 0.07 mL of bovine milk XO prepared immediately before use. After pre-incubation at 25 °C for 15 min, the reaction was initiated by the addition 0.66 mL of substrate solution into the mixture. The assay mixture was then incubated at 25 °C for 15 min. The reaction was stopped by adding 0.2 mL of 0.5 N HCl, and the absorbance was measured at 295 nm using a Thermo Scientific Evolution 260 spectrophotometer (Thermo Scientific, Waltham, MA). The half-maximal inhibitory concentration (IC_{50}) was generated from the absorbance data.

Determination of antioxidant capacity

The antioxidant capacities of the honey samples were determined using three different assays; TPC, ferric reducing antioxidant power and free radical scavenging activity of DPPH.

TPCs were determined using the Folin–Ciocalteau procedure with gallic acid as standard[28]. Briefly, 20 μL samples (1 mg/mL), 400 μL of 0.2 N Folin–Ciocalteu reagent and 680 μL of distilled water were mixed, and the mixture was vortexed. Following 3-min incubation, 400 μL of Na_2CO_3 (10%) solution was added and vortexed. After vortexing, the mixture was incubated with intermittent shaking for 2 h at 25 °C. Absorbance was measured at 760 nm at the end of the incubation period. TPC concentration was calculated as mg of gallic acid equivalents per gram sample, using a standard graph.

Working FRAP reagent was obtained as required by mixing 25 mL acetate buffer (300 mM, pH 3.6), with 2.5 mL of 10 mM TPTZ solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution[29]. Next, 100 μL of the honey sample was mixed with 3 mL of freshly prepared FRAP reagent and incubated for 4 min at room temperature. Absorbance was read at 593 nm against reagent blank. $FeSO_4{\cdot}7H_2O$ was used a positive control to construct a reference curve (31.25–1000 μM, $r^2 = 0.999$), FRAP values were expressed as μmol $FeSO_4{\cdot}7H_2O$ equivalent of g sample.

The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was used to determine the radical scavenging activity of the honey samples. The colorimetric test was performed using the Molyneux method[30]. For each sample, various concentrations of 0.75 mL of extracts of honey were mixed with 0.75 mL of 0.1 mM of DPPH in methanol. Radical scavenging activity was measured using Trolox as standard, and the values were expressed as SC_{50} (mg sample per mL), the concentration of samples causing 50% scavenging of DPPH radicals.

Table 1. Data from studies included in the identification markers.

Honey code	Flora	Location	Dom.pollen%	Major familia	Predominant pollen
H1	Chestnut	Zonguldak	70	*Fagaceae*	*Castaneasativa* Mill.
H2	Chestnut	Trabzon	68	*Fagaceae*	*Castaneasativa* Mill.
H3	Chestnut	Giresun	66	*Fagaceae*	*Castaneasativa* Mill.
H4	Chestnut	Rize	72	*Fagaceae*	*Castaneasativa* Mill.
H5	Oak	Kırklareli	62	*Fagaceae*	*Quercusrobur* L.
H6	Oak	Kırklareli	52	*Fagaceae*	*Quercusrobur* L.
H7	Oak	Kırklareli	60	*Fagaceae*	*Quercusrobur* L.
H8	Oak	Bolu	55	*Fagaceae*	*Quercusrobur* L.
H9	Polyfloral	Aydın	N.D.	*Verbenaceae, Fagaceae, Rosaveae, Lamiaceae, Fabaceae, Rutaceae*	*
H10	Polyfloral	Bolu	N.D.	*Malvaceae, Fabaceae, Fagaceae asteraceae, Alliaceae*	*
H11	Polyfloral	Giresun	N.D.	*Fagaceae, Ericaveae, Rocaceae, Fabaceae, Tiliaceae*	*
H12	Polyfloral	Kars	N.D.	*Fabaceae, Lamiacea, Betulaceae, Apiaceae, Campanulaceae, Papaveraceae*	*

ND, not detected.
*Dominant pollen was not present.

Table 2. IC_{50} values of each enzyme and antioxidant properties of honey samples*.

Honey types	Honey code	Urease-IC_{50} (g/mL)	Xanthine oxidase-IC_{50} (g/mL)	Total phenolics (mg GAE/100 g sample)	FRAP (μmol $FeSO_4{\cdot}7H_2O$/g sample)	DPPH-SC_{50} (mg/mL)
Chestnut	H1	0.034 ± 0.001^{g}	0.039 ± 0.000^{a}	41.170 ± 0.412^{e}	3.545 ± 0.036^{f}	40.866 ± 0.409^{d}
	H2	0.010 ± 0.000^{a}	0.028 ± 0.000^{a}	52.672 ± 1.053^{g}	4.336 ± 0.087^{h}	41.350 ± 0.827^{d}
	H3	0.034 ± 0.001^{g}	0.033 ± 0.000^{a}	38.900 ± 0.960^{d}	3.111 ± 0.078^{e}	40.874 ± 1.022^{d}
	H4	0.025 ± 0.000^{f}	0.030 ± 0.000^{a}	65.300 ± 1.306^{i}	4.690 ± 0.094^{i}	$35.100 \pm 0.702^{c,d}$
Oak	H5	0.012 ± 0.000^{b}	0.101 ± 0.002^{b}	62.260 ± 0.623^{h}	4.247 ± 0.085^{h}	18.350 ± 0.367^{b}
	H6	0.014 ± 0.000^{c}	$0.110 \pm 0.001^{b,c}$	57.674 ± 1.154^{g}	4.669 ± 0.117^{i}	10.892 ± 0.272^{a}
	H7	0.021 ± 0.000^{e}	0.147 ± 0.004^{c}	36.806 ± 0.972^{d}	3.104 ± 0.062^{e}	$38.160 \pm 0.763^{c,d}$
	H8	0.018 ± 0.000^{d}	$0.135 \pm 0.010^{b,c}$	45.460 ± 0.909^{f}	3.680 ± 0.037^{g}	32.100 ± 0.321^{c}
Polyfloral	H9	0.045 ± 0.001^{h}	0.384 ± 0.004^{e}	10.193 ± 0.204^{a}	0.990 ± 0.025^{b}	250.555 ± 6.263^{g}
	H10	0.052 ± 0.001^{i}	0.255 ± 0.006^{d}	29.480 ± 0.737^{c}	1.895 ± 0.038^{d}	76.153 ± 1.523^{e}
	H11	0.069 ± 0.001^{j}	0.452 ± 0.005^{f}	9.400 ± 0.188^{a}	0.527 ± 0.013^{a}	411.416 ± 10.285^{h}
	H12	0.076 ± 0.001^{k}	0.361 ± 0.083^{e}	19.500 ± 0.390^{b}	1.440 ± 0.014^{c}	152.200 ± 1.522^{f}

*Means ± standard deviations; Different letters (a–k) in the same columns are significantly different at the 5% level ($p < 0.05$)

Table 3. ''Paired samples-*t*'' test correlation coefficients.

	Paired differences	Urease-IC_{50}	Xanthine oxidase-IC_{50}	Total phenolics	FRAP	DPPH-SC_{50}
Urease-IC_{50}	Pearson Correlation	1.000	0.804†	−0.839†	−0.881†	0.741†
	Sig. (two-tailed)		0.000	0.000	0.000	0.000
	N	36.000	36.000	36.000	36.000	36.000
Xanthine oxidase-IC_{50}	Pearson Correlation	0.804†	1.000	−0.863†	−0.907†	0.864†
	Sig. (two-tailed)	0.000		0.000	0.000	0.000
	N	36.000	36.000	36.000	36.000	36.000
Total phenolics	Pearson Correlation	−0.839†	−0.863†	1.000	0.979†	−0.826†
	Sig. (two-tailed)	0.000	0.000		0.000	0.000
	N	36.000	36.000	36.000	36.000	36.000
FRAP	Pearson Correlation	−0.881†	−0.907†	0.979†	1.000	−0.851†
	Sig. (two-tailed)	0.000	0.000	0.000		0.000
	N	36.000	36.000	36.000	36.000	36.000
DPPH-SC_{50}	Pearson Correlation	0.741†	0.864†	−0.826†	−0.851†	1.000
	Sig. (two-tailed)	0.000	0.000	0.000	0.000	
	N	36.000	36.000	36.000	36.000	36.000

†Correlation is significant at the 0.01 level (two-tailed).

Statistical analysis

The results were given in the form of arithmetical mean values and standard deviations. The SPSS 13.00 for Windows software package was used for the statistical analysis of the gathered data (SPSS Inc., Chicago, IL). Significance of the analysis of the results was based on the Kruskal–Wallis test and Pearson correlation. Significant differences were statistically considered at the level of $p < 0.05$.

Results and discussion

The purpose of this study was to determine whether honey exhibits an inhibitory effect on two anti-inflammatory enzymes of vital importance to human health. Common inhibitors of both enzymes will be important to alternative medicine as protective agents and in the treatment of gastric ulcer and gout. IC_{50} values for each enzyme in the analyzed honey samples are given in Table 2.

Before examining enzyme inhibition, the antioxidant capacities of the honeys used as inhibitor sources were clarified. Three different methods were used to evaluate the antioxidant capacity of the honeys; TPC, the ferric reducing antioxidant assay (FRAP) reflecting total antioxidant capacity and the DPPH assay showing total radical scavenging capacity. TPC of the honeys varied widely, from 9.400 to 65.000 mg GAE/100 g sample (Table 2). Oak and chestnut honeys had higher level of TPC concentrations which showed the statistically significant differences from each other, polyfloral honeys exhibited the lowest TPC. A positive correlation was determined between TPC and FRAP values (r^2: 0.979, $p < 0.01$), and between TPC and DPPH activity (r^2: −0.826, $p < 0.01$) (Table 3). Higher TPC indicates higher antioxidant capacity, as well as DPPH radical scavenging activities.

Previous studies have reported that chestnut and oak honeys are both dark colored and have higher phenolic contents and associated antioxidant capacity than light-colored honeys[17,19,31]. Seventy to eighty percent of the dry weight of honey consists of carbohydrate and 2% of secondary metabolites The bioactivity of honey derives mainly from these secondary metabolite agents in its structure, that varies depending on the floral sources involved[32,33]. Phenolic compounds, vitamins (A, E and C), free amino acids, proteins and enzymes represent the secondary metabolites of honey. The great majority of these compounds that give rise to the true quality of honey are phenolic structure molecules, phenolic acids, flavanols, pro-anthocyanins and tannins that determine the aroma, taste and sensory characteristics of honey[34,35]. These secondary metabolites possess not only anti-oxidant activities, but also anti-microbial, anti-tumoral, and anti-inflammatory functions[36–38].

All three honeys in the study inhibited urease and XO in a manner dependent on the varying IC_{50} values and concentrations. Oak honey exhibited the highest degree of inhibition of urease, followed by chestnut honey and polyfloral honey. The inhibitory value of oak honey was approximately 3 times greater than that of polyfloral honey.

The high urease inhibitor activity of oak honey may derive from its high phenolic composition. Indeed, a negative correlation was determined between TPC and urease enzyme inhibition (r^2: −0.839, $p < 0.01$) (Table 3). This significantly high correlation derives from the presence of phenolic compounds in oak honey. A previous study of ours reported that oak honey is rich in rutin, gallic acid and protocatechuic acid[19]. Also, Modolo et al.[39] stated that some flavonoids as rutin, quercetin, and luteolin are effective in the *H. pylori* inhibition with IC_{50} values of 11.2 μM, 67.6 μM, and 35.5 μM, respectively. In another study, the IC_{50} values of *Eucalyptus grandis* stem and bark methanolic extracts ranged between 6.5 and 50.0 mg/mL[40].

Similarly to urease, a negative correlation was also determined between honey TPC levels and XO inhibitions (r^2: −0.863, $p < 0.01$) (Table 3). Compared to oak honey, chestnut had a low inhibitory effect on XO and exhibited lower IC_{50} inhibition values. A previous study of ours reported that chestnut honey is rich in quercetin, caffeic acid, coumaric acid and protocatechuic acid[19]. One of the study reported that caffeic acid and its species are a significant inhibitor for XO[6], and another reported that chestnut honey exhibits XO inhibition[17].

In conclusion, honeys studied in this research particularly with their high levels of TPC may be considered as a good nutrition source for the inhibition of the enzymes urease and XO. Regular consumption of these products may contribute to a reduction in several forms of ROS-mediated pathological injury.

Declaration of interest

This study was supported by the KTU-BAP Project (Project number: 970). The author reports no financial or other conflict of interest relevant to the subject matter of this article.

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