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Effect of honey on oxidation, chlorination and nitration by purified equine myeloperoxidase

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ABSTRACT

Objective: To evaluate the antioxidant effect of honey using two classical methods generally used, and for the first time to test the effect of honey on the oxidation, chlorination and nitration by purified equine myeloperoxidase (MPO).
Methods: The antioxidant activity of three Algerian honey samples (nectar honey, mixed honey and honeydew honey) was evaluated by two classical methods, the ferric- reducing/antioxidant power (FRAP) assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity.
Results: Honeydew honey had the highest reducing power and DPPH radical-scavenging activity, whereas nectar honey showed the lowest reducing power and DPPH radical-scavenging activity. All honey samples showed a significant inhibitory effect on the chlorination activity of equine MPO, but honeydew honey was the weakest inhibitor. The three samples were poorly inhibitor on the MPO oxidation and nitration activities, except for nectar honey that exerted an inhibitory effect at the highest tested concentration of 10%. These later results seem to contradict those obtained with DPPH and FRAP.
Conclusions: The antioxidant capacity of honey is mainly due to the phenolic compounds and flavonoids it contained. It has been suggested that MPO might be involved in the antioxidant, not pro-oxidant, activity of phenolic compounds.

1. Introduction

The concept "antioxidant" is usually linked to free radical scavenging and that reactive oxygen species are essentially harmful and should be eliminated. However, the purpose of the "antioxidant defense network" is not to remove all reactive oxygen species, but to control their levels so as to allow useful functions whilst minimising oxidative damage[1]. According to Papineni and Orton[2], intraperitoneal administration of honey in a rabbit model significantly enhanced the myeloperoxidase (MPO) activity. In most of publications, the higher level of MPO was connected to enhanced free radicals productions[3,4].

However, some observations expand this view and show that MPO-derived oxidants are critically involved in a more subtle modulation of signaling pathways[5].

It has been also suggested that MPO might be involved in the antioxidant, not pro-oxidant, activity of phenolic compounds[6]. Peroxidases mediate *in vitro* the pro-oxidant or antioxidant activity of phenolic compounds, depending on the chemical environment[7]. It has been demonstrated that chlorinated flavonoids were more efficient than their parent compounds in modulating neutrophils' oxidative burst[8].

The antioxidant properties of honey have been extensively studied, but there are important discrepancies in the published data. Indeed, honey is a very complex mixture, containing a number of ingredients that are involved in oxidant/antioxidant physiological processes, such as hydrogen peroxide, nitrite, nitrate, glucose, glucose oxidase, iron, copper, chlorine, iodine, catalase, tyrosine, tryptophan, arginin, flavonoids and phenolic acids[9].

It has been suggested that catalase originating from pollen, nectar, or microorganisms would be responsible for the enzymatic H_2O_2-neutralizing activity of honey[10,11]. However, according to Kwakman and Zaat[12], catalase has never been identified in honey. Interestingly, peroxidases are among the most abundant proteins in petunia nectar and the level of H_2O_2 accumulation in tobacco and petunia nectar is inversely related to the level of peroxidase activity

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in these nectars[13]. Possibly nectar-derived peroxidases rather than catalase might be a cause of variation in H_2O_2-neutralizing capacity of different honeys.

Besides the direct antimicrobial effects of honey[14], research has also focused on identification of the substances responsible for its anti-inflammatory[15,16] and its immunomodulatory effects[17,18]. It has been proposed that the antioxidant capacity of honey is mainly due to the phenolic compounds and flavonoids it contained and there is a high correlationship between polyphenols and honey antioxidant capacity, if possible a synergistic effect is observed on honey polyphenols and the more than 181 compounds that form part of honey[19].

Therefore, the aim of this study is to evaluate the antioxidant effect of honey using two methods generally used, and for the first time to test the effect of honey on the oxidation, chlorination and nitration by equine MPO.

2. Materiel and methods

2.1. Reagents

Analytical grade sodium and potassium salts, hydrogen peroxide (H_2O_2, 30%w/v) were from Merck (VWRI, Belgium). 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), ethanol, ascorbic acid, $FeCl_3$, trichloroacetic acid, and potassium ferricyanide were all purchased from Sigma (represented by the Algerian Chemical Society, Algeria). Trypan blue was from ICN Biomedicals (Solon, OH, USA). Tyrosine was from Acros (Geel, Belgium). Ethylene diamine tetra acetic acid (EDTA), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2-nitro-5-thiobenzoic acid, taurine, catalase, diammonium salt (ABTS), percoll, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (Bornem, Belgium). Microtitration plates (Cliniplate EB) and white combiplates were from Fisher Scientific (Aalst, Belgium). All aqueous solutions were prepared with water previously purified in a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Honey samples

Three samples of Algerian honey, namely honeydew honey (HH: darker honey), mixed honey (MH) and nectar honey (NH: lighter honey) were obtained directly from beekeepers, belonging to different geographical regions. Raw honeys used in this study were not submitted to thermal treatments or pasteurization, or any other operation able to alter natural composition. Honey samples were stored at 4 °C in the dark until further analysis. The botanical origin, color and accordance with international standards of the three varieties of honey were confirmed by aspecialist laboratory (CARI ABSL Laboratory, Louvain La Neuve, Belgium).

2.3. Blood collection and isolation of neutrophils

Blood samples were drawn from healthy horses by jugular vein puncture in 9 mL Vacutainer® tubes with EDTA (1.6 mg/mL blood) as anticoagulant. The horses were clinically healthy: they were fed, bred and housed under identical conditions and were not under medical treatment (Faculty of Veterinary Medicine, University of Liege, Belgium). PMNs were isolated according to the technique previously described by Pycock *et al.*[20] on a discontinuous density gradient of Percoll in Hank's balanced salt solution (HBSS) buffer formed by a 85% solution, overlaid by a 70% solution. The anticoagulated whole blood, laid on the top of the gradient, was centrifuged at 400 ×*g* for 20 min at 20 °C. The PMNs were collected at the interface between the two gradient layers and washed in two volumes of physiological saline solution. The cell pellets were suspended in 20 mmol/L phosphate-buffered saline at pH 7.4 containing 137 mmol/L NaCl and 2.7 mmol/L KCl. The cell preparation was ⩾ 96% neutrophils, with a cell viability of 97% as measured by the Trypan blue exclusion test. Each batch of neutrophils was obtained from 60 mL blood drawn from one horse, the cells were used immediately after isolation, the experiment was completed within 5 h and each assay was performed in triplicate. Each experiment was repeated at least twice with different cell batches.

2.4. Purification of equine MPO

The purification of equine MPO was previously described in detail[21]. In brief, MPO was extracted from isolated equine neutrophils and purified by two chromatographic steps (ion exchange and gel filtration) to reach a purity of > 98% (as established by electrophoresis with enzymatic detection on electrophoretic bands).

2.5. DPPH radical scavenging activity

The DPPH radical scavenging effect (H/e_ transferring ability) of honey samples was measured as per the method described by Chen *et al.*[22]. The DPPH was dissolved in absolute ethanol to a 0.2 mmol/L concentration. A 100 µL aliquot of honey solution (0.1 g/mL) was diluted to 500 µL with 70% ethanol, and vigorously mixed with 400 µL of DPPH solution by vortexing. The mixture was incubated at room temperature for 15 min and the absorbance of the solution was measured at 517 nm. Results were expressed as mg ascorbic acid equivalents (AAE)/100 g.

Sample blank (B1) consisted of 600 µL of 70% ethanol and 400 µL of DPPH whereas DPPH blank (B2) contained 100 µL of honey sample, 500 µL of 70% ethanol and 400 µL of absolute ethanol. The DPPH scavenging activity was calculated using the following formula:

DPPH scavenging activity (%) = $\{1 - [(T1 - B2)/B1]\} \times 100$

where T1, B1 and B2 are the absorbencies of the sample, sample blank and DPPH blank, respectively.

2.6. Ferric reducing/antioxidant power (FRAP) assay

The reducing power of the ethanolic extracts of honey was determined according to the method of Oyaizu[23]. A 1 mL aliquot of ethanolic honey extract (10% v/v) was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. After this, 2.5 mL of 10% trichloroacetic acid was mixed by vortexing. The mixture was centrifuged at 3000 r/min for 10 min. A 2.5 mL aliquot of the supernatant was mixed with an equal amount of milli Qwater and 0.5 mL of 0.1% $FeCl_3$. The absorbance was measured at 700 nm using a spectrophotometer. Precipitation or flocculation was never observed. Assays were performed in triplicate. Ascorbic acid (1.0 mg/mL) was used as a reference standard. The increase in absorbance provided an indication of higher reducing power of the samples being analysed.

2.7. *In vitro* effect of honey on equine MPO oxidation, nitration and chlorination activities

For this part of our work, we used the techniques as described by Kohnen *et al.*[24].

2.7.1. Nitration activity of MPO on tyrosine

The experiments were carried out at pH 5.5 in acetate buffer (100 mmol/L) with tyrosine (1.5 mmol/L), equine MPO (1 µg/mL or

200 mU/mL), NaCl (150 mmol/L), H_2O_2 (1 mmol/L) and $NaNO_2$ (5 mmol/L). Reactions were performed for 30 min at 37 ˚C in the presence of diluted honey at the final concentration of 2%, 5% or 10%. The formation of 3-nitrotyrosine was monitored by UV-visible spectroscopy at 405 nm (Multiskan Ascent, Thermo Labsystem, Helsinki, Finland) after alkalinization with 100 µL NaOH 0.1 mol/L. Assay without honey was taken as positive control (100% nitration activity).

2.7.2. Chlorination activity of MPO on taurine

Taurine is a good substrate for the chlorination activity of MPO, yielding chlorotaurine of which the concentration is evaluated by a subsequent reaction with 2-nitro-5-thiobenzoate (TNB) absorbing at 412 nm. The TNB solution was prepared as follows: 1 mmol/L 5,5'- dithiobis(2-nitrobenzoic acid), 5 mmol/L EDTA and 20 mmol/L $NaBH_4$ were dissolved in phosphate buffer (50 mmol/L) at pH 6.6 and allowed to react at 37 ˚C for 30 min. The concentration of TNB was directly estimated by UV-visible spectroscopy at 412 nm (ε_{412} = 13 600 M^{-1} cm^{-1}). For the chlorination reaction, 10 µL of taurine (150 mmol/L), 10 µL of equine MPO (0.1 µg/mL, *i.e.*, 20 mU), 20 µL NaCl (1.5 mol/L) and diluted honey at concentration of 2%, 5% or 10% were added in the wells of a 96-well microtitration plate. The volume was adjusted to 190 µL with 100 mmol/L acetate buffer (pH 5.5), and 10 µL of 10 mmol/L H_2O_2 solution was added. The plate was incubated for 30 min at 37 ˚C. Then, 35 µL of catalase (3 mg/mL) was added to stop the reaction and the solution was incubated again for 15 min at 37 ˚C. Finally, 100 µL of the TNB solution (0.45 mmol/L) were added and TNB reaction with chlorotaurine was followed by UV-visible spectroscopy (Multiskan Ascent Thermo Labsystem, Helsinki, Finland) at 405 nm (the closest available filter to 412 nm). Assay without honey was taken as positive control (100% chlorination activity).

2.7.3. Oxidation activity of MPO on ABTS

ABTS is converted by the peroxidasic activity of MPO into the stable cation radical form $ABTS^{•+}$, which strongly absorbs around 700 nm. The experiments were carried in acetate buffer at pH 5.5 with the following reagents: ABTS (750 µmol/L), equine MPO (0.5 µg/mL, *i.e.*, 100 mU/mL), NaCl (150 mmol/L), H_2O_2 (0.5 mmol/L), $NaNO_2$ (5 mmol/L), and diluted honey at the final concentration of 2%, 5% or 10%. The solution was incubated for 30 min at 37 ˚C, and the formation of $ABTS^{•+}$ was monitored by UV-visible spectroscopy at 690 nm (Multiskan Ascent Thermo Labsystem, Helsinki, Finland). Assay without honey was taken as positive control (100% oxidation activity).

2.8. Statistical analysis

DPPH and FRAP were determined in triplicate. In terms of the enzymatic model within an experiment, each point was repeated three times and each experiment was repeated three times with cell batches from different horses, so that the *n* value of one experimental point was 9. Data were given as mean ± SD and statistical analysis was performed with GraphPad InStat 3.05 (GraphPad Software, San Diego, CA, USA). A *P* value < 0.05 was considered significant.

3. Results

3.1. FRAP assay and DPPH radical scavenging activity

We found that HH had the highest reducing power and DPPH radical-scavenging activity, which may be related to the high phenolic and flavonoid contents, whereas NH showed the lowest reducing power and DPPH radical-scavenging activity (Table 1).

Table 1
Results of DPPH radical scavenging activity and FRAP assay.

Honey samples	DPPH radical-scavenging activity (mg EAA ± SD)	Reducing power (µmol/L FeII) (mg/100 g ± SD)
NH	21.87 ± 3.66	59.44 ± 0.78
MH	25.50 ± 4.24	70.78 ± 1.16
HH	32.22 ± 6.80	551.50 ± 1.52

3.2. Effect of honey samples on equine MPO oxidation, nitration, and chlorination activities

All honey samples showed a significant inhibitory effect *vs.* positive control on the chlorination activity of equine MPO, but HH was the weakest inhibitor. The three samples were poorly inhibitor *vs.* positive control on the MPO oxidation and nitration activities, except for NH that exerted an inhibitory effect at the highest tested concentration of 10% (Figures 1–3).

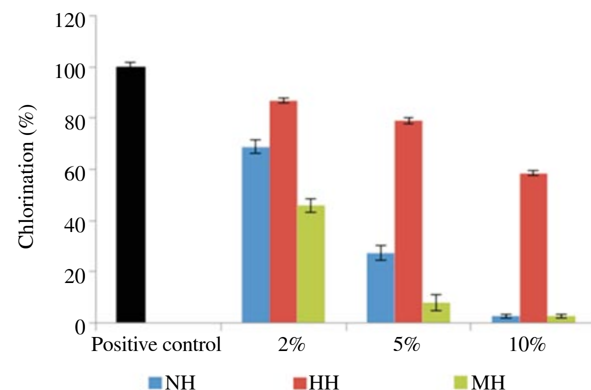

Figure 1. Inhibiting effect of honey sample on the chlorination activity of equine MPO on taurine.
The assay is based on the production of chlorotaurine produced by the MPO/H_2O_2/Cl^- system in the presence of various concentrations (2%, 5% and 10%) of each honey. Honey samples showed a very significant inhibitory effect on the chlorination activity of equine MPO (two-tailed *P* value < 0.000 1, mean value ± SD; *n* = 9), but HH was the weakest inhibitor.

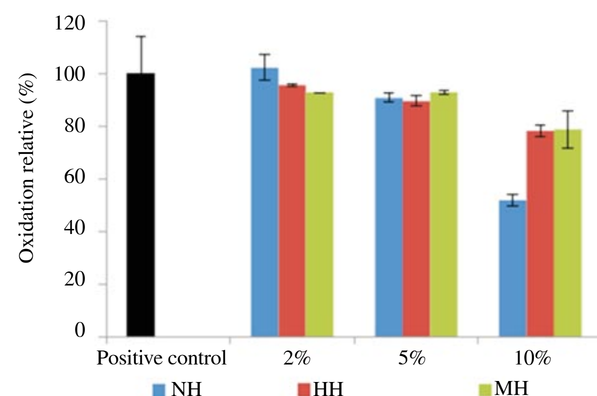

Figure 2. Inhibitory effect of honey sample on the oxidation activity of equine MPO on ABTS.
The assay is based on the conversion of ABTS by peroxidasic activity of MPO into the stable cation radical form ($ABTS^{•+}$) in the presence of various concentrations (2%, 5% and 10%) of each honey sample. The three samples were poorly inhibitor on the MPO oxidation activity, except for NH that exerted a very significant inhibitory effect at the highest tested concentration of 10% (Two-tailed *P* value = 0.004 1, mean value ± SD; *n* = 9).

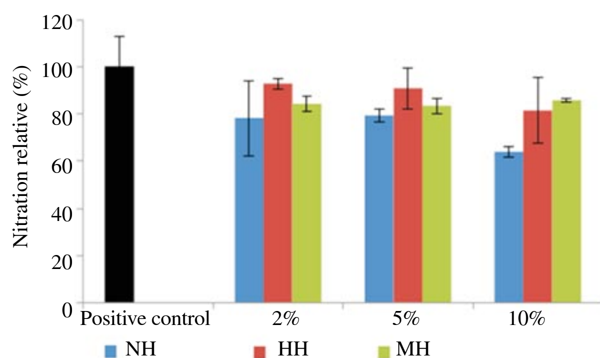

Figure 3. Inhibitory effect of honey sample on the nitration activity of equine MPO ontyrosine.
The assay was based on the formation of 3-nitrotyrosine by the MPO/H_2O_2/Cl^-/NO_2^- system, in the presence of various concentrations (2%, 5%, 10%) of each honey. All samples were poorly inhibitor on the MPO oxidation activity, except for NH that exerted a very significant inhibitory effect at the highest tested concentration of 10% (two-tailed *P* value = 0.0086, mean value ± SD; *n* = 9).

4. Discussion

Numerous tests have been developed for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively. The antioxidant capacity depends on the assay method and the radical source used[25].

DPPH is an unwavering, nitrogen-centered free radical that has been widely employed to test the free radical scavenging ability of various samples, including honeys. In evaluating the radical scavenging potential of honeys, DPPH scavenging activity indicates superior antioxidant activity[26]. FRAP is a simple, direct test that is widely used to test antioxidant capacity; this test estimates the amount antioxidants or reductants present in a sample based on their ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) compounds[27]. According to Moniruzzaman *et al.*[28], the use of DPPH assay coupled with various other useful methods such as FRAP are preferred because they are able to reflect the antioxidant properties of honey more accurately. In a previous studies of us[29], using the same samples, we have shown that HH had the highest contents of phenolics and flavonoids and the darkest color, meanwhile NH had the lowest contents of phenolics and flavonoids and the lightest color. In the present study, HH exhibited the highest DPPH radical-scavenging activity and fraps value and NH the lowest.

Results from several studies show that darker honeys possess stronger antioxidant activity than lighter ones[30,31]. It has been demonstrated a strong correlation between the contents of phenolic compounds in honeys from various floral sources and their antioxidant capacities and antibacterial activities[32-34]. The darker the honey, the higher its phenolic content and its anti-oxidative power[35,36]. It has also been shown that, HHs possess higher antioxidant capacities than nectar ones[37,38].

In the present study, *in vitro* enzymatic model was used to represent biological conditions. All honey samples showed a significant inhibitory effect on the chlorination activity of equine MPO, but HH was the weakest inhibitor. The three samples were poorly inhibitor on the MPO oxidation and nitration activities, except for NH that exerted an inhibitory effect at the highest tested concentration of 10% (Figures 2 and 3). These results seem to contradict those obtained with DPPH and FRAP.

However, the reaction between flavonols and HOCl may be more complicated than a simple oxidant-antioxidant interaction, and phenolic compounds can also react with HOCl to form stable chlorinated components[39]. Chlorinated flavonols may mimic estrogens cardioprotective properties and quercetin chlorinated derivatives were shown to exhibit significantly greater antioxidant capacity than the unmodified quercetin. Its chlorination enhances both inhibition of low density lipoprotein oxidation and total radical antioxidant potential, as well as plays a role in cardioprotection[40].

In honey, quercetin is one of the more representative polyphenols[41]. Boersma *et al.*[42] reported that formation of chlorinated and nitrated isoflavones with greater antioxidant capabilities is enhanced by NO_2^- via MPO dependant pathway, honey contains $NO2^-$[39-43] proposed that both chlorination and nitration imply the involvement of NO_3^- in the process. Honeys also contain appreciable amounts of NO_3^-, higher in HHs than in NH[44].

In general, chlorinated flavonoids are more effective than their parent compounds, revealing that these unexplored flavonoids present promising scaffolds to be developed and may result in new agents that can be used as useful tools for the anti-inflammatory therapy. In this regard, the therapeutic potential of flavonoids' metabolites, and in this particular case, the chlorinated flavonoids, should not be neglected[8,44].

In this study, the results with DPPH and FRAP and those obtained with the enzymatic model seem contradictory. However, the process by which honey exerts its antioxidant effect has not yet been fully elucidated. It has been argued that peroxidases mediate *in vitro* the pro-oxidant or antioxidant activity of phenolic compounds, depending on the chemical environment, and honey might offer such an environment.

Conflict of interest statement

We declare that we have no conflict of interest.

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