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Effect of Honey on Hydroxyl Radical Generation, Glutathione Depletion And on Myeloperoxidase Released in the Extra-Cellular Milieu by Activated Neutrophils

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Abstract: Using Electron spin resonance we have shown that (1): nectar honey (NH) and mixed honey (MH) at 2% and 5% (w/v)generate the hydroxyl radical (OH) via Fenton-like reactions and noticed a partial quenching of this radical at 10% (w/v). Honeydew honey (HH) produced partial quenching at 2%, strong quenching of OH at 5% (w/v) and complete quenching at 10% (w/v). (2): the three honey types honey exhibited a biphasic activity on glutathione (GSH) depletion, as they lead to GSH depletion and increase reactive oxygen species (ROS) generation at low concentrations, but prevent this depletion at higher concentrations. The present study investigates also the effects of honey on the release of myeloperoxidase (MPO) by equine neutrophils (PMNs) activated with phorbol 12-myristate 13-acetate (PMA), we noticed also a biphasic effect. At low concentration the three honeys increased significantly the total MPO released in the extra-cellular milieu by activated PMNs but decreased it significantly at higher concentrations. Honey is a very complex mixture containing a number of ingredients involved in "oxidant/antioxidant» physiological processes,bifunctional activities may occur at different dosages.

Key words: Honey · Hydroxyl Radical · Myeloperoxidase · Neutrophils

INTRODUCTION

Although the basal production of ROS can be beneficial to maintain the redox homeostasis, still the excessive production of ROS, followed by a depletion of endogenous antioxidant enzymes, remains responsible for the disruption of the redox state balance named “oxidant stress”. Among these species, $^{\bullet}OH$ a potent oxidizing agent capable of reacting with a wide variety of targets over very short distances has been implicated in different models of neutrophil-mediated tissue injury.$^{\bullet}OH$ cytotoxic effects have been shown for both prokaryotic and eukaryotic cells; bacteria, yeast and human cells [1-3]. For example, $^{\bullet}OH$ production from H_2O_2 by neutrophil granules is a first line of defense against bacteria during acute inflammation. Similarly, the H_2O_2-induced injury in *Escherichia coli* appears to be mediated by $^{\bullet}OH$ via Fenton reaction [2,4]. $^{\bullet}OH$ reacts indiscriminately with most metabolites and macromolecules, in many cases generating other radicals in the process [1].

During the inflammatory process, phagocytosis by PMNs is highly correlated with the respiratory burst and often accompanied by neutrophils degranulation and the extracellular release of enzymes such as MPO. MPO is also found to a much lesser extent in monocytes and some macrophages [5]. MPO has long been considered a key constituent of the neutrophil’s cytotoxic armament by catalyzing the formation of hypochlorous acid, a potent oxidant that displays bactericidal activity *in vitro* [6]. Although the generation of oxidants by MPO is beneficial

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in terms of the immune response to invading pathogens, inappropriate stimulation of oxidant formation by this enzyme (wrong place, wrong time, excessive levels) can result in host tissue damage [7].

Most of publications the higher level of MPO was connected to enhanced free radicals productions. However, some observations expand this view and show that MPO-derived oxidants are critically involved in a more subtle modulation of signaling pathways [8]. It has been also suggested that MPO might be involved in the antioxidant, not pro-oxidant, activity of phenolic compounds in endothelial Cells under oxidative stress [9]. Peroxidases mediate *in vitro* the pro-oxidant or antioxidant activity of phenolic compounds, depending on the chemical environment [10].

Reduced GSH, a thiol-containing tripeptide, is a significant contributor for maintaining the intracellular redox state and, as such, is an important component of the overall cellular defensive mechanisms against ROS. An important function of this intracellular antioxidant is to scavenge ROS produced during normal aerobic cellular respiration; if left unchecked, such ROS could oxidize and, thereby, damage nucleic acids, proteins and lipids [11]. Several studies have noted greater depletions of intracellular GSH in cancer, than in normal, cells upon their exposures to polyphenols. Normal cells maintain a proper intracellular redox status with their antioxidant enzymes and their sufficient supply of reduced GSH and thus are less susceptible to cytotoxic damage by pro-oxidant polyphenols [12].

It is widely accepted that honey is beneficial to health, thanks to its antioxidant properties among all of its beneficial aspects. Explanation(s) remain obscure and the mechanism by which it acts also so far remains to be elucidated. The antioxidant activity of honey has been extensively studied, but there are remarkable discrepancies in the published data. The main cause and far from being negligible is that honey is a very complex mixture containing a number of ingredients involved in "oxidant/antioxidant" physiological processes [13]. 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 immunomodulatory effects [17-19]. It has been proposed that the antioxidant capacity of honey is due mainly to the phenolic compounds and flavonoids they contain 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 [20].

The concept “antioxidant” is usually linked to free radical scavenging and that ROS are essentially harmful and should be eliminated. However, the purpose of the ‘‘antioxidant defense network” is not to remove all ROS, but to control their levels so as to allow useful functions whilst minimising oxidative damage [21]. Honey seems to either reduce or activate the production of reactive oxygen species from neutrophils. Honey and its components are able to either stimulate or inhibit the release of certain cytokines (tumor necrosis factor-α, interleukin-1β, interleukin-6) from human monocytes and macrophages. Honey seems also, to either reduce or activate the production of reactive oxygen species from neutrophils. Similarly, human keratinocytes, fibroblasts and endothelial cell responses (e.g., cell migration and proliferation, collagen matrix production, chemotaxis) are positively affected in the presence of honey. However, the immunomodulatory activity of honey is highly complex because of the involvement of multiple quantitatively variable compounds among honeys of different origins [22].

The aim of this work was to evaluate the effect of three types of raw Algerian honey on hydroxyl radical produced by the Fenton reaction and glutathione depletion by using Electron Spin Resonance-spin trapping and on MPO Released in the Extra-Cellular Milieu by Activated Neutrophils (MPO-ELISA Assay). At the best of our knowledge the two latter studies have never been achieved so far in the case of honey.

MATERIALS AND METHODS

Reagents: 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), paranitrophenyl phosphate, ethylene diamine tetra-acetic acid (EDTA), phorbol 12-myristate 13-acetate (PMA),Percollwere purchased from Sigma (Bornem, Belgium), $FeSO_4$ was purchased from Merck.Horseradish peroxidase (HRP), diethylenetetraminepenta acetic acid (DTPA) were purchased from Boehringer Mannheim, Trypan blue was from ICN Biomedicals, Inc (Ohio, USA). Analytical-gradNaCl, KCl, H2O2 30%, were supplied by Merck (VWRI, Leuven, Belgium). all aqueous solutions were prepared with water previously purified in a Milli-Q water system (Millipore, Bedford, MA, USA

Honey Samples: Three samples of Algerian honey, honeydew honey (HH), mixed honey (MH1) and nectar honey (NH), were directly obtained from beekeepers, belonging to different geographical regions. Raw honeys used in this study were not submitted to thermal

treatments or to pasteurization or any other operation able to alter natural composition. Honey samples were stored at 4°C in the dark until further analysis. The three varieties of honey are in accordance with international standards (CARI ABSL, Belgium, which performed the analysis).

Blood Collection and Isolation of PMNs: Blood samples were drawn from healthy horses by jugular venipuncture in 9 ml vacutainer tubes with EDTA (1.6 mg/mlblood) as anticoagulant. The horses were clinically normal; they were fed, bred and housed under identical conditions and not under medical treatment (Faculty of Veterinary Medicine, University of Liège, Belgium). PMNs were isolated following the technique of Pycock *et al*. [23] on a discontinuous density gradient of Percoll formed by a 1.108 g/ml solution, overlaid with a 1.1087 g/ml solution. The anticoagulated whole blood, laid on the top of the gradient, was centrifuged at 400g for 20 min at 20°C. The PMNs were collected at the interface between the 2 gradient layers and washed in 2volumes of physiological saline solution. The cell pellets were suspended in 20 mM phosphate buffer saline (PBS) at pH 7.4 containing 137 mMNaCl and 2.7 mMKCl. The cell preparation was =96% neutrophils with a 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 5hours and each assay was performed in triplicate. Each experiment was repeated at least twice with different cell batches.

Before the experiments, neutrophils (10^6 cells/ml PBS) were incubated with diluted honey samples (2, 5, 10 and 20%) and the cell viability was estimated by the trypan blue exclusion test in order to exclude a cytotoxicity of the honey.

Electron Spin Resonance (ESR)-spin Trapping Experiments: Electron spin (or electron paramagnetic) resonance (ESR) is a spectroscopic technique that detects unpaired electrons present within the sample. As such, ESR is the only accurate approach that can provide direct evidence for the presence of a free radical. In addition, the analysis of the ESR spectrum generally enables the determination of the identity of the free radical [24]. Because of their short lifetime, the detection of such free radicals is very difficult in biological samples and therefore, spin trap agents (nitrones) are used to form adducts with a higher lifetime.

Effect of honey on hydroxyl radical produced by the Fenton reaction.

H_2O_2 (5 µl, 10^{-3} M) in the presence of $FeSO_4$(25µl, 10^{-4} M), DTPA (25 µl, 10^{-4}M), H_2O_2 (5 µl, 10^{-3}M) and DMPO (50 µl, 100 mM) were added to each honey sample at the final concentration of 2, 5 or 10% (w/v). The reaction mixtures were immediately transferred into the flat cell in the TM cavity of the spectrometer. ESR spectra were recorded at room temperature on a Bruker spectrometer (Bruker ESP300E, Bruker, Kalsruhe, Germany) operating at X-band frequency (9.8 GHz) and at non-saturating microwave power (20 mW).The instrumental settings were the following: 100 KHz modulation frequency, 1.012 G modulation amplitude, 3480 G magnetic field center and receiver gain was 2.10^4. The sweep width was 100 G and the total number of scans was 6. The hyperfine splitting constants were measured from the experimental spectra by means of a Bruker Win-Simfonia program running under Microsoft Windows. The Fenton reaction in the absence of honey sample was considered as a positive control (CtrL) for DMPO-OH adducts and the signal height of its ESR spectrum was taken as 100%.

EPR Spin Trapping Investigation of the Effect of Honey on the Thiyl Radical Generation: The reaction mixtures 25mg/mL HRP, 10mM GSH, 1 mM H_2O_2 and 100 mM DMPO in phosphate buffer (pH 7, 5) were added to each honey sample at final concentrations of 2% and 10% and immediately transferred into the flat cell in the TM cavity of the spectrometer. ESR spectra were recorded at room temperature on a Bruker spectrometer (Bruker ESP300E, Bruker, Kalsruhe, Germany) operating at X-band frequency (9.8 GHz) and at non-saturating microwave power (20 mW).). The instrumental conditions were as follows : microwave power, 20 mW ; receiver gain,2x 10^4 ; time constant, 164 ms ; time conversion, 40,96 ms ; center field, 3480± 50 G and number of scan,2. The hyperfine splitting constants were measured from the experimental spectra by means of a Bruker Win-Simfonia program running under Microsoft Windows. The EPR signal, corresponding to the spin adduct of DMPO/thiyl (GS●) resulted from the reaction of DMPO with the GS● radicals produced by the HRP enzymatic activity on GSH. The EPR signal obtained in the absence of honey was taken as the control spectrum (CtrL).

Effect of honey on Total MPO Released in the Extra-Cellular Milieu by Neutrophils Activated with PMA (MPO-ELISA Assay): The neutrophil suspensions (10^6cells/mL) were incubated for 10 min at 37 °C in

darkness with honey at final concentrations of 2, 5, 10 and 20% (w/v) for each honey sample and then activated for 30 min at 37 °C once again in darkness with PMA at the final concentration of 0.8 µM. After activation, the cell suspensions were centrifuged for 10 min (450 *g*) and the supernatants were collected. To measure the total MPO released by activated neutrophils in the extra-cellular milieu, an original Equine MPO ELISA assay designed by Franck *et al*. [25] was performed, using a specific kit provided by BiopTis (Liège, Belgium). Briefly, polyclonal antibodies against equine MPO were obtained in rabbit and coated on 96 wells-microtiter plates. The supernatants, which contained MPO released by the cells, were diluted 200-fold with PBS, loaded into the wells and incubated at 4 °C overnight. After washing, a second polyclonal antibody against equine MPO, raised in guinea pigs and labeled with alkaline phosphatase, was loaded into the wells and incubated for 2 h in darkness. After an ultimate washing, the wells were loaded with a solution of paranitrophenyl phosphate for the measurement of phosphatase activity and incubated for 30 min at 37 °C in darkness. The absorbance (405 nm) proportional to the content of MPO in the wells was read with Multiscan Ascent (Thermo Scientific). The control was performed with neutrophils activated with PMA in presence of PBS instead of honey and was taken as 100% MPO release to compare with the effects of honey.

Statistical Analysis: Determination the effect of honey on total MPO released by PMA-activated PMNs was done in triplicate. The n value of one experimental point was 5. Data are given as mean ± SD and statistical analysis was performed with Graph Padins tat 3.05 (Graph Pad Software, San Diego California, USA). A p value < 0.05 was considered as significant.

RESULTS

Effect of Various Types of Honey on Hydroxyl Radical Produced by the Fenton Reaction: The Fenton reaction is based on the reduction of H_2O_2 by an electron donated by metal ions to produce hydroxyl radical (•OH). Iron is used as an electron donor. We used the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO), which reacts with •OH to form a relatively stable paramagnetic species, the DMPO-OH spin adduct with a characteristic four-line ESR spectrum (quartet with 1:2:2:1 intensities) [24]. Figure 1a and 1b spectrum 1 shows the ESR spectra obtained from the trapping of hydroxyl radicals (•OH) by DMPO in the absence of honey samples (CtrL). The addition to the complete system of 2% (w/v) of NH resulted in more complex spectra with an increased intensity of the four-line ESR signal attributed to the DMPO-OH adducts (sign Ø spectrum 2in Fig. 1b) and a new signal (sign * spectrum 2in Fig 1b). Similar data were obtained with MH, but are not presented here. When, 5% of NH (w/v) were added to the complete system, there was no real modification of the shape of the ESR signal intensity attributed to DMPO-OH adducts and the new signal as shown in Fig. 1a spectrum 3. Upon addition of 10% of NH we noticed a decrease of the height of the spectrum VsCtrL. In contrast, the lines attributed to the unidentified species were enhanced (Fig 1a spectrum 4). The addition of HH at 2 % (w/v) already resulted in a slight decrease of the 4-line ESR spectrum of DMPO-OH adduct (sign Ø in Fig 1b spectrum 2), the new signal was also present. When high concentrations of HH (5 and 10% w/v) were added to the mixture, the lines corresponding to DMPO-OH adducts were strongly reduced and even totally disappeared for the highest concentration of 10 %. In contrast, the second ESR signal (*) enhanced dramatically (Fig. 1b spectra 3 and 4).

EPR Spin Trapping Investigation of the Effect of Honey on the Thiyl Radical Generation: The peroxidase-catalysed oxidation system allowed investigation of the formation of the thiyl (GS●) radical intermediate triggered by the addition of H_2O_2 in the presence of a peroxidase (HRP). This system was designed to assess the capability of honey to protect glutathione from the oxidant attack by the H_2O_2/HRP couple (Fig 2). Fig (2a) shows EPR spectrum characteristic of the thiyl radical produced by the enzymatic system HRP/GSH/H_2O_2 in the presence of DMPO (control). The addition to the complete system of 2% (w/v) of HH and NH resulted in an increased intensity of the four-line ESR signal attributed to the thiyl radical adduct, MH being slightly active (Fig 2b).The four-line EPR spectrum was totally abolished when HH, NHand MHat final concentration of 5%was added to the reaction mixture (Fig 2c).

Effect of Honey on Total MPO Released in the Extra-Cellular Milieu by Neutrophils Activated with PMA (MPO-ELISA Assay): The MPO amount released by activated PMNs (Ctrl PBS), in which PBS was used instead of honey, was set as 100% MPO release (Ctrl). The addition OF NH, HH and MH honey at the final concentration of 2% increased respectively the MPO release by 96,76%;64,48% and 15,53%. The addition of NH and HH honey at the final concentration of 5% also increased respectively the MPO release by 47, 59% and 28,71%, while no significant effect was observed for

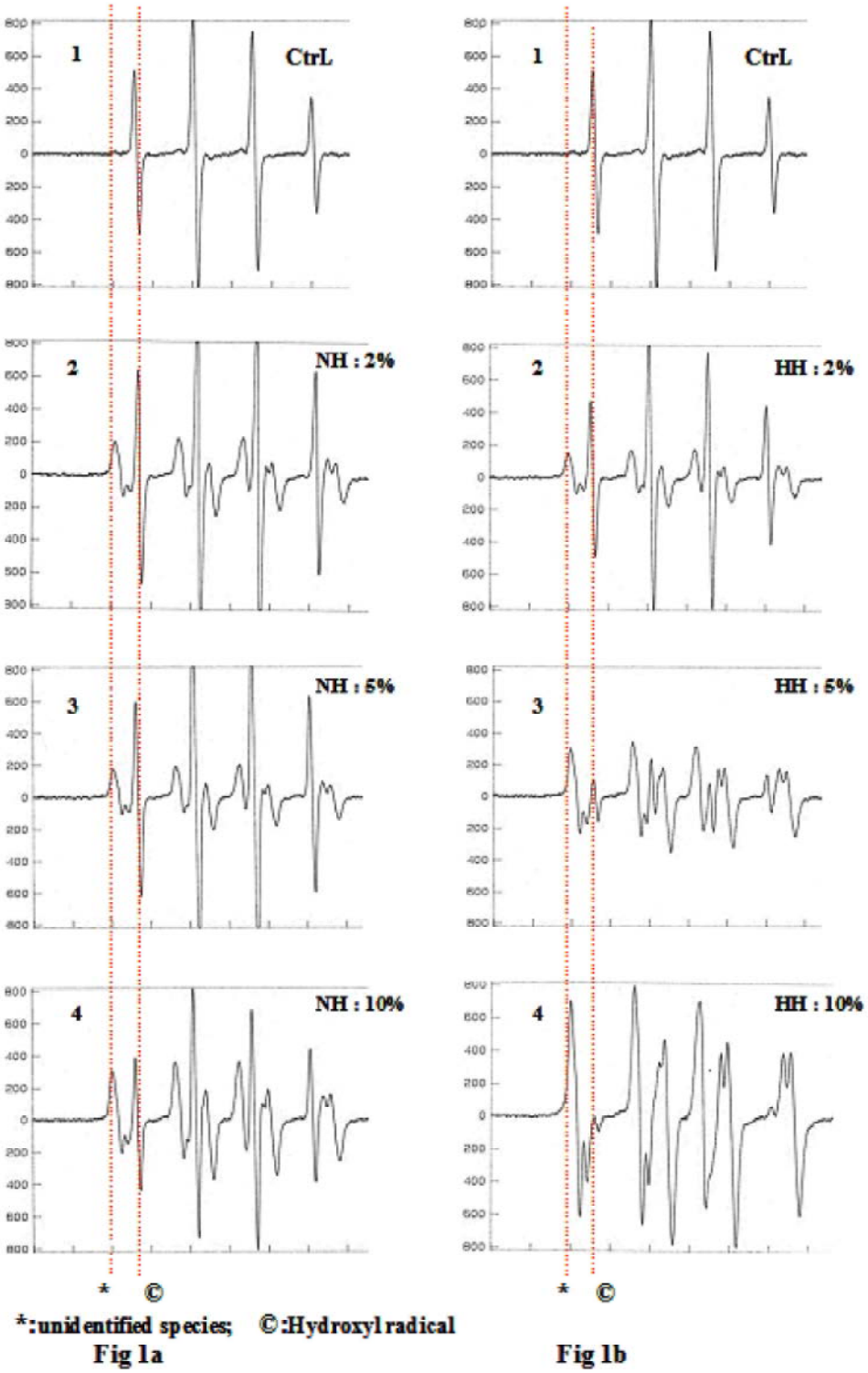

Fig. 1: Hydroxyl radical (˙OH) by fenton reaction (Fe^{2+}/DTPA/H_2O_2) and effects of honey
(Fig 1a) ESR spectra obtained from the trapping of hydroxyl radicals (•OH) by DMPO in the absence of honey samples (Ctrl). (1b): same as (1a) but with addition of honey at final concentration of 2% (w/v).(1c): same as (1a) but with addition of honey at final concentration of 5% (w/v).).(1): same as (1a) but with addition of honey at final concentration of 10% (w/v)The instrument settings are listed in materiel and methods

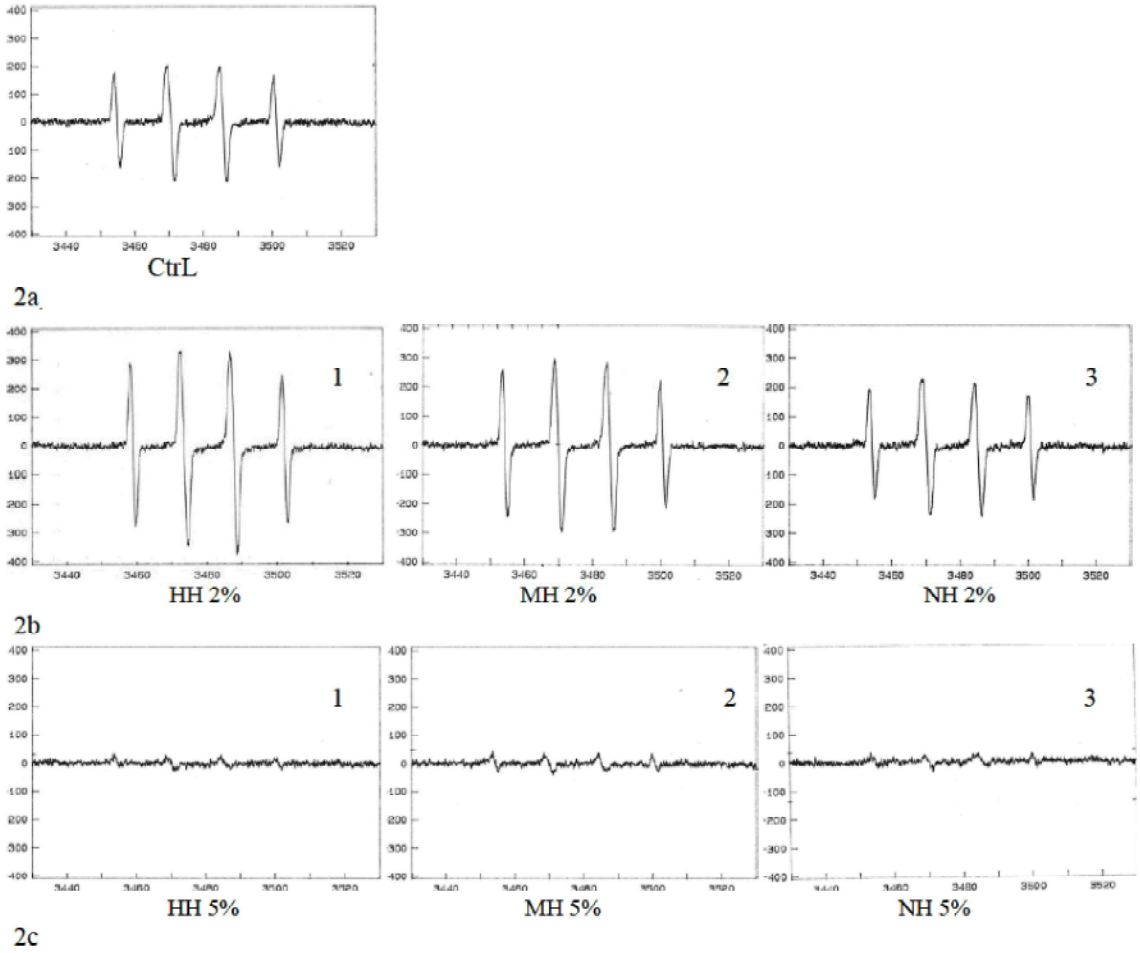

Fig. 2: Thiyl (GS●) radical produced by the enzymatic system (HRP/GSH/H_2O_2) and effects of honey.
(2a): Complete system.HRP/GSH/H_2O_2 + DMPO. (2b): same as (2a) but with addition of honey at final concentration of 2% (w/v).(2c): same as (2a) but with addition of honey at final concentration of 5% (w/v).The instrument settings are listed in materiel and methods.

MH at the same concentration. Meanwhile the addition of NH, HH and MH honey at the final concentration of 10% and 20% decreased respectively the MPO release by 12,7% ; 50%; 30,55% and 60.21; 59.96; 59.33.

DISCUSSION

In the present work we found that NH and MH honeys at 2% and 5% (w/v) generate •OH via Fenton-like reactions with generation of unidentified species Fig 1a spectrum 2 and 3). In1994, Mason *et al*. [24] ascertained that since the Fenton reaction requires hydrogen peroxide, a substance that catalyzes hydrogen peroxide formation would increase the signal. It is well established that honeys contain glucose oxidase enzyme which catalyses the formation of hydrogen peroxide, after honey dilution[26]. Since the rates of hydrogen peroxide production increase with honey concentrations until the optimal dilution at which honey produces the maximal amounts of hydrogen peroxide (which is between 40 and 60%) [26],and since •OH generation increases with honey dilution [27],it would thus be expected an increase of the DMPO-OH signal intensity when the concentrations of honey increased.

Therefore, 10 % NH (w/v) were added to the complete system and the shape of the ESR spectrum was monitored. In contrary, we noticed apartial quenching of •OH, but the lines attributed to the unidentified species were enhanced (Fig 1a spectrum 4).

Addition of HH produced partial quenching at 2%, strong quenching of OH at 5% (w/v) and complete quenching at 10% (w/v), the signal attributed to the new

species enhanced dramatically. The other radical species observed could not be unequivocally identified Fig 1b specta 2, 3 and 4).

Alvarez-Suarez *et al*. [28] previously noticed that all Cuban honeys studied generated •OH to varying extents via the Fenton reaction. While in another study carried out by the same authors, it was found on the contrary that all the studied Cuban honeys scavenged the •OH generated via the Fenton reaction [29]. In a similar study Henriques *et al*. [30], studying three types of honey (Manuka honey, pasture honey P59 and artificial honey), reported that only pasture honey P59 was able to generate free radicals. These authors concluded that additional unidentified species might arise from secondary reactions involving free radicals and the organic components of honey. It must be noticed that these authors used only a single dilution. Honeys were shown to cause oxidative damage that restricted bacterial growth and caused cytotoxic degradation of DNA due to the formation of •OH and appear to involve some components in honey that have not yet been identified [27,31].

As, the other radical species observed could not be unequivocally identified, we cannot state here if the quenching of •OH may represent an antioxidant or prooxidant effect of honey. Honey's therapeutic properties are largely attributed to its antimicrobial and anti-oxidant activities. Bactericidal activity could be due to •OH and possibly to these secondary organic radicals. Any antibacterial properties of honey will thus be a balance between the opposing activities of radical generation and quenching [30].

GSH is an intracellular antioxidant which accounts for over 90% of the intracellular non-protein thiols [32]. It is commonly viewed as an universal free radical scavenger and major intracellular antioxidant [33]. In a *vivo* studies, honey was found to maintain or enhance the level of non-protein sulfhydyl substances (such as GSH) [34]. Similar observation was made by Korkmaz *et al.* [35]) and Galal *et al*. [36]. In agreement with what precedes,it appears from our results that at 5% (w/v) the three honey types prevent GSH depletion (Fig 2b).

While strategies for increasing GSH levels were developed to increase cellular defenses and resist toxicity, strategies for depleting GSH were developed to increase the sensitivity of tumors and certain parasites to radiation, drugs or endogenous killing mechanisms [37]. Apoptosis is a conserved homeostatic process critical for organ and tissue morphogenesis, development and senescence. This form of programmed cell death also participates in the etiology of several human diseases including cancer, neurodegenerative and autoimmune disorders Although

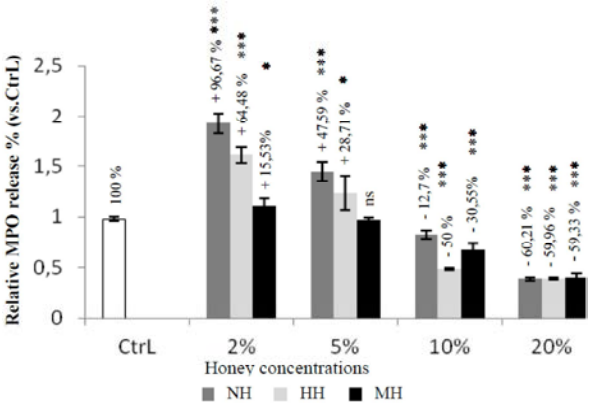

Fig. 3: Effect of honey on Total MPO Released in the Extra-Cellular Milieu by Neutrophils Activated with PMA (MPO-ELISA Assay
Columns and bars represent mean ± standard deviation of the results of five measurements. *Significant difference (*P < 0.05, **P < 0.01, ***P<0.001) vs. CtrL. + : enhancement;-inhibition; ns: no significant

initial studies suggested that GSH depletion was only a byproduct of oxidative stress generated during cell death, recent discoveries suggest that GSH depletion plays a critical as regulator of apoptosis [38].

According to Jaganathan and Mandal. [39], honey at low concentration (3% v/v) induced apoptosis of the HCT-15 and HT-29 colon cancer cells by causing the depletion of intracellular non-protein thiols and increasing the reactive oxygen species (ROS) generation.In line with these findings, we have also shown in the present study that honey at low concentrations causes a depletion of GSH and increase ROS generation (Fig 2c).

Compared to CtrL, the three honey samples at 10% and 20% (w/v) decreased significantly the total MPO released in the extra-cellular milieu by activated PMNs (Fig 3).

Very few studies reported the effect of honey on MPO even though Medhi *et al.* [40] reported that Manuka Honey reduced the MPO level in rats induced ulcerative colitis. Honey was also found to decrease the luminol-enhanced chemiluminescence in opsonisedzymosan-stimulated whole blood and isolated leukocytes [41]. But, as was the case for GSH depletion, at the best of our knowledge there is not available published data on the effect of honey on the release of MPO from activated PMNs.

Surprisingly, except for MH at 5% the three honey types at 2% and 5% increased significantly the total MPO released in the extra-cellular milieu by activated PMNs (Fig 3).

However, upon its release from neutrophils, the enzyme becomes inactive very frequently in tissue microenvironment. Therefore, both enzymatically active MPO and enzymatically inactive MPO are present at inflammatory sites [42].

According to Papineni and Orton [43] after, intraperitoneal administration of a single dose honey 0,1 ml (50% v/v) in athymic nude mice, significant enhancement in MPO activity was observed within 3 hrs-probably after that honey has been over diluted by corporal fluids-with robust PMNs activation at different lymph nodes. According to Lau *et al*. [8] independently of his catalytic action MPO exerts leukocyte-activating functions, an event reminiscent of other PMN-derived potent pro-inflammatory cytokines such as TNF-α and IL-8.

Which should attract attention in this study is that the honey presents a "pro-oxidant" effectat such low concentration. In another context, it have been shown previously that honey at 1% (w/v) significantly enhanced the expression of MMP-9 mRNA in primary cultures of human keratinocytes [17] and stimulate human monocytic cells to produce inflammatory cytokines (e.g., TNF-α, IL-6 and IL-1β) important in resolution of infection and tissue repair [44].

Similarly, it has been shown that honey at low concentration stimulates the immune system; in particular, the multiplication of β-lymphocytes and T-lymphocytes [45], increases phagocytic activity [45-46] and induces the chemotactic activity of isolated neutrophils [47] The stimulatory activity of honey at low concentration is probably due to a low level of an inflammatory/stimulatory mediator is honey [17, 45].

Virtually no research has been conducted on the biphasic effect of honey except that it has been shown by Tsiapara *et al.* [48] that honey exhibited a biphasic activity in breast cancer MCF-7 cells depending on the concentration-an antiestrogenic effect at low concentrations and an estrogenic effect at high concentrations.

CONCLUSION

The present findings suggest that honey exhibit a biphasic activity on hydroxyl radical generation, glutathione depletion and MPO release by activated neutrophils. Being "pro-oxidant" at low concentration and "antioxidant" at high concentration in a variable extent depending on honey type. Thus, by acting as an antioxidant and pro-oxidant, honey may produce both beneficial and adverse effects important in the prevention and pathogenesis of disease.. The mechanisms by which honey affects the release of MPO and the GSH depletion need to be clarified. However, our results herein may represent framework for further studies. Nevertheless, the immunomodulatory activity of honey is highly complex because of the involvement of multiple quantitatively variable compounds among honeys of different origins.

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REFERENCES

1. Halliwell, B., 1995. The biological significant of oxygen-derived species. J.S. Valentine, C.S. Foote, A. Greenberg and J.F. Liebman (Eds.), Active Oxygen in Biochemistry, Blackie Academic and Professional, Glasgow, pp: 313-335.
2. Imlay, J.A. and S. Linn, 1988. DNA damage and oxygen radical toxicity. Science, 240: 1302-1309.
3. Perrone, G.G., S.X. Tan and I.W. Dawes, 2008. Reactive oxygen species and yeast apoptosis. Biochim. Biophys. Acta, 1783: 1354-1368.
4. Imlay, J.A., S.M. Chin and S. Linn, 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*. Science, 240: 640-642.
5. Davies, M.J., C.L. Hawkins, D.I. Pattison and M.D. Rees, 2008. Mammalian heme peroxidases: from molecular mechanisms to health implications. Antioxid Redox Signal, 10(7): 1199-1234.
6. Winterbourn, C.C., 2002. Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid Toxicology, pp: 181-182, 223-227.
7. Michael, J., 2011. Davies. Myeloperoxidase-derived oxidation: mechanisms of biological damage and its prevention. J Clin Biochem Nutr. Jan; 48(1): 8-19.
8. Denise Lau, Hanke Mollnau, Jason P. Eiserich, Bruce A. Freeman, Andreas Daiber, Ursula M. Gehling, Jens Brümmer_, Volker Rudolph, Thomas Münzel, Thomas Heitzer, Thomas Meinertz and Stephan Baldus, 2005. Myeloperoxidase mediates neutrophil activation by association with CD11b_CD18 integrins. PNAS, 102(2): 431-436

9. Lee, S.J., G.I. Mun, S.M. An and Y.C. Boo, 2009 Antioxidant effect of p-coumaric acid in endothelial cells exposed to high glucose plus arachidonic acid BMB reports, pp: 561.
10. Chan, T.S., G. Galati, A.S. Pannala, C. Rice-Evans and P.J. O'Brien, 2003 Simultaneous detection of the antioxidant And pro-oxidant activity of dietary polyphenolics in a peroxidise system. Free Radic. Res., 37: 87-794.
11. Moskaug, J.O., H. Carlsen, M.C. Myhrstad and R. Blomhoff, 2005. "Polyphenols and glutathione synthesis regulation," The American Journal of Clinical Nutrition, 71: 16985-17025, View at Scopus.
12. Harvey Babich, Alyssa G. Schuck, Jeffrey H. Weisburg and Harriet L. Zuckerbraun, 2011 .Research Strategies in the Study of the Pro-Oxidant Nature of Polyphenol Nutraceuticals. Journal of Toxicology.
13. Ahmed, M., S. Aissat and N. Djebli, 2012. How Honey Acts as an Antioxidant? Medicinal Aromatic Plants, 1: 1-2.
14. Kwakman, P.H., A.A. teVelde, L. De Boer, D. Speijer, C.M. Vandenbroucke-Grauls and S.A. Zaat, 2010. How honey kills bacteria. FASEB J., 24: 2576-2582.
15. Kassim, M., M. Achoui, M. Mansor and K.M. Yusoff, 2010. The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin E(2) in inflammatory tissues. Fitoterapia, 81: 1196-1201.
16. Van den Berg, A.J., E. van den Worm, H.C. van Ufford, M.J. Halkes, M.J. Hoekstra and C.J. Beukelman, 2008. An *in vitro* examination of the antioxidant and anti-inflammatory properties of buckwheat honey. J. Wound Care, 17: 172-178.
17. Majtan, J., P. Kumar, T. Majtan, A.F. Walls and J. Klaudiny, 2010. Effect of honey and its major royal jelly protein 1 on cytokine and MMP-9 mRNA transcripts in human keratinocytes. Exp. Dermatol., 19: e73-e9.
18. Ranzato, E., S. Martinotti and B. Burlando, 2012. Epithelial mesenchymal transition traits in honey-driven keratinocyte wound healing: comparison among different honeys. Wound Repair Regen, 20: 778-785.
19. Tonks, A.J., R.A. Cooper, K.P. Jones, S. Blair, J. Parton and A. Tonks, 2003. Honey stimulates inflammatory cytokine production from monocytes. Cytokine, 21: 242-247.
20. Elizabeth Pérez-Pérez, Patricia Vit and FazlulHuq, 2013. Flavonoids and polyphenols in studies of honey antioxidant activity. Int. J. Med. Plant Altern. Med., 1(4): 063-072, April 2013.
21. Halliwell, B., 2008. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and *in vivo* studies? Archives of Biochemistry and Biophysics, 476: 107-112.
22. Juraj Majtan, Honey, 2014. An immunomodulator in wound healing. Wound Rep Reg., 22: 187-192.
23. Pycock, J.F., W.E. Allen and T.H. Morris, 1987. Rapid, single-step isolation of equine neutrophils on a discontinuous Percoll density gradient. Res. Vet. Sci., 42: 411-412.
24. Mason, R.R., P.M. Hanna, M.J. Burkitt and M.B. Kadiiska, 1994. Detection of Oxygen-derived Radicals in Biological Systems Using Electron Spin Resonance. Environ Health Perspect, 102(S 10): 33-36.
25. Franck, T., S. Grulke, G. Deby-Dupont, C. Deby, H. Duvivier, F. Peters and D. Serteyn, 2005. Development of an enzyme-linked immunosorbent assay for specific equine neutrophil myeloperoxidase measurement in blood. J. Vet. Diagn. Invest, 17: 412-419.
26. Bang, L.M., C. Bunting and P.C. et Molan, 2003. The effect of dilution on the rate of hydrogen peroxide production in honey and its implications for wound healing. J. Altern. Compl. Med., 9: 267-73.
27. Brudzynski, K. and R. Lannigan, 2012. Mechanism of honey bacteriostatic action against MRSA and VRE involves hydroxyl radicals generated from honey's hydrogen peroxide. Front Microbiol., 3: 36.
28. Alvarez-Suarez, J.M., S. Tulipani, D. Díaz, Y. Estevez, S. Romandini, F. Giampieri, E. Damiani, P. Astolfi, S. Bompadre and M. et Battin, 2010. Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. Food Chem. Toxicol., 48: 2490-2499.
29. José, M., 2012. Alvarez-Suarez and Francesca Giampieri and ElisabettaDamiani and Paola Astolfi and Daniele Fattorini and Francesco Regoli and José L. Quiles and Maurizio Battino.Radical-scavenging Activity, Protective Effect Against Lipid Peroxidation and Mineral Contents of MonofloralCuban Honeys. Plant Foods Hum Nutr., 67: 31-38.
30. Henriques, A., S. Jackson, R. Cooper and N. Burton, 2006. Free radical production and quenching in honeys with wound healing potential. Journal of Antimicrobial Chemotherapy, 58: 773-777.

31. Brudzynski, K., K. Abubaker, L. St-Martin and A. Castle, 2011. Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey. Front Microbiol., 2: 213.
32. Baruchel, S., G. Bounous and P. Gold, 1994. Place for an antioxidant therapy in human immunodeficiency virus (HIV) infection. J. Nutr., 112: 1747-1755.
33. Grigory, G. Borisenko, Ian Martin, Qing Zhao, Andrew A. Amoscato_, Yulia Y. Tyurina and Valerian E. Kagan, 2004. Glutathione Propagates Oxidative Stress Triggered by Myeloperoxidase in HL-60 Cells. J. Biol. Chem., 279: 23453-23462.
34. Al Swayeh, O.A. and A.T.M.M. Ali, 1998. Effect of ablation of capsaicin sensitive neurons on gastric protection by honey and sucralfate. Hepato-Gastroenterol., 45: 297-302.
35. Asli Korkmaz and Dürdane Kolankaya, 2009. Anzer honey prevents N-ethylmaleimide-induced liver damage in rats. Experimental and Toxicologic Pathology, 61: 333-337.
36. Reem, M. Gallal, Hala F. Zaki, Mona M. Seif El-Nasr and M. Azza, 2012. Agha. Potential Protective Effect of Honey Against Paracetamol-induced Hepatotoxicity. Archives of Iranian Medicine, 15: 11.
37. Angel, L., 2011. Ortega, Salvador Mena and Jose M. Estrela. Glutathione in Cancer Cell Death. Cancers, 3: 1285-1310; doi: 10.3390/cancers3011285.
38. Franco, R. and J.A. Cidlowski, 2009. Apoptosis and glutathione: beyond an antioxidant. Cell Death and Differentiation, 16: 1303-1314.
39. Jaganathan and Mandal, 2010. Saravana Kumar Jaganathan and MahitoshMandal. Antiproliferative Effects of Honey and of Its Polyphenols: A Review. Journal of Biomedicine and Biotechnology. Volume 2009, Article ID 830616, pp: 13.
40. Medhi, B., A. Prakash, K. AvtiP, N. SaikiaU, P. Pandhi and K.L. Khanduja, 2008. Effect of Manuka honey and sulfasalazine in combination to promote antioxidant defense system in experimentally in duced ulcerative colitis model in rats. Indian Journal of Experimental Biology, 46: 583-590.
41. Mesaik, M.A., M.K. Azim and S. Mohiuddin, 2008. Honey modulates oxidative burst of professional phagocytes. Phytotherapy Research, 22(10): 1404-1408.
42. Kumar, A.V., 2010. Sharma. Neutrophils: Cinderella of innate immune system. International Immunopharmacology, 10: 1325-1334.
43. Papineni, R.V.L. and S. Orton, 2012. Intraperitoneal Administration of Honey Elicit Robust Luminescence Signals from Myelo,peroxidase Activation. Presentation at World Molecular Imaging Congress Dublin, Ireland September 5-8, 2012http://fr.scribd.com/doc/100813301/ Honey-Medicinal-Value-and-Mechanism-Dr-Rao-Papineni.
44. Tonks, A.J., E. Dudley, N.G. Porter, J. Parton, J. Brazier, E.L. Smith and A. Tonks, 2007. A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. J. Leukoc Biol., 82(5): 1147-55.
45. Nizar Abuharfeil, Rateb Al-ORan and Mahmoud Abo-Shehada, 1999. The Effect of Bee Honey on the Proliferative Activity of Human B-and T-Lymphocytes and the Activity of Phagocytes. Food and Agricultural Immunology, 11(2): 169-177.
46. Shehab Ahmed Lafi, Huda R. Sabar Al-Dulaimy and Muntaha M. Al-Aloosi, 2012. Honey depots phagocytosis *in vitro* Anb. Med. J., 10(1): 13-17.
47. MayukoMiyagawa, Miki Fukuda, Yuriko Hirono, Ayaka Kawazoe, Eri Shigeyoshi, Masaaki Sakura, Toru Takeuchi, Osamu Mazda, Kent E. Pinkerton and Minoru Takeuchi, 2010. Effect of Jungle honey on the chemotactic activity of neutrophils. Journal of ApiProduct and ApiMedical Science, 2(4): 149-154.
48. Tsiapara, A.V., M. Jaakkola, I. Chinou, K. Graikou, T. Tolonen, V. Virtanen and P. Moutsatsou, 2009. Bioactivity of Greek honey extracts on breast cancer (MCF-7), prostate cancer (PC-3) and endometrial cancer (Ishikawa) cells: Profile analysis of extracts. Food Chem., 116: 702-708.