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Antiacanthamoebic properties of natural and marketed honey in Pakistan

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ABSTRACT

Objective: To determine antiacanthamoebic activity of natural and marketed honey samples.

Methods: Natural honey samples were collected directly from the bee hive and marketed honey samples were purchased from the local market in Karachi, Pakistan. Both honey samples were tested for their flavonoid content (quercetin equivalent per gram of the extract) and phenolic content (gallic acid equivalent per gram). Furthermore, their antioxidant activity was determined by measuring 2,2-diphenyl-1-picrylhydrazyl. Using amoebistatic and amoebicidal assays, the effects of honey samples were tested against growth and viability of *Acanthamoeba* parasites.

Results: Natural honey exhibited potent amoebistatic and amoebicidal effects, in a concentration-dependent manner. Honey-treated *Acanthamoeba castellanii* showed loss of acanthopodia, following which amoebae detached, rounded up, reduced in size, decreased in cytoplasmic mass and they were observed floating in the culture medium. Importantly, honey-treated amoebae did not revive when inoculated in fresh growth medium, however, glycerol-treated amoebae exhibited viable trophozoite and active growth. In contrast, marketed honey samples varied in their efficacy against *Acanthamoeba castellanii*. The proportion of flavonoid, as determined by quercetin measurements and the proportion of phenolic, as determined by gallic acid measurements was higher in natural honey compared with marketed honey. Similarly, the antioxidant activity, as determined by 2,2-diphenyl-1-picrylhydrazyl scavenging activity was higher in natural honey *vs.* marketed honey.

Conclusions: This study shows that natural honey has antiacanthamoebic properties and possesses higher flavonoid, phenolic and antioxidant properties compared with the marketed honey. These findings are of concern to the public, health officials, and to the manufacturers regarding production of honey for medical applications.

1. Introduction

Honey has been used as a medicine since ancient times in many cultures and communities. The major constituent of honey is carbohydrates, especially fructose and glucose (85%–95% of total sugars) [1], while other components present in minor quantities include organic acids, amino acids, proteins, enzymes, lipids, flavonoids and vitamins that are responsible for its multiple biological properties such as, wound healing, antibacterial effects against a wide range of pathogenic bacteria [2,3], antifungal [4,5], antiviral [2,3], antioxidant [6,7], antitumour [8] activities and various skin disorders [2,9]. Antioxidants such as polyphenols and flavonoids are effective in reducing the risk of heart disease, cancer, inflammatory processes, asthma, infected wounds, chronic wounds, skin ulcers, and cataracts [2–10]. This may explain widespread use of honey resulting in its production commercially, artificially, and through natural bee hive. However, the composition and antioxidant capacity of honey

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depends on various factors, principally the plant source used by the honey bees. Despite its broad-spectrum activities against a range of bacterial pathogens, honey has not been tested against protozoan pathogen, *Acanthamoeba*. *Acanthamoeba castellanii* (*A. castellanii*) is a free-living amoeba that is known to produce cutaneous infections, blinding keratitis and fatal encephalitis [11–13]. In the present study, we determined antiacanthamoebic activity of natural honey collected directly from the bee hive and compared its effects with the marketed honey samples, both of them are accessible to the local community. Antioxidant properties (polyphenols and flavonoids) of natural *vs.* marketed honey were determined further.

2. Materials and methods

2.1. Source of honey samples

For natural honey, two different samples were collected directly from two different bee hives at the Rajanpur District of Southern Punjab, Pakistan. The samples were stored in the laboratory at room temperature until further analysis. For marketed honey, commonly used honey samples were purchased from the local market in Karachi, Pakistan (Table 1).

2.2. Determination of flavonoid in natural and marketed honey

Flavonoid content was determined as previously described [14]. Briefly, a 2-mL solution of the test material (1 g/mL) was added to an equal volume of 2% $AlCl_3 \cdot 6H_2O$ in methanol. The mixture was vigorously shaken and absorbance was read at 367 nm after 10 min of incubation. Flavonoid content is expressed as mg of quercetin equivalent per gram of the extract.

2.3. Determination of phenolic content

Phenolic content was determined as previously described [15]. Briefly, 1 mL of Folin-Ciocalteu reagent was added to the extract solution (1 g/mL) and final volume adjusted to 46 mL by addition of distilled water. After 3 min, 3 mL of 2% Na_2CO_3 was added. Subsequently, the mixture was placed on a shaker for 2 h at room temperature and finally absorbance was recorded at 760 nm. Phenolic content is expressed as mg of gallic acid equivalent per gram of the test material.

2.4. Antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The reducing power and free radical scavenging activity of test samples were determined using DPPH assay as previously described [14]. DPPH is a known radical and scavenger for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of the reaction. Because of a strong absorption band centred at about 520 nm, the DPPH radical has a deep violet colour in solution, and it becomes colourless or pale yellow when neutralized. This property allows visual monitoring of the reaction. Briefly, test samples of honey (0.5–200.0 mg/mL) and the reference antioxidant, ascorbic acid (0.005–500.000 μg/mL) was dissolved in distilled water for free radical scavenging activity. A 0.1-mmol/L solution of DPPH radical in methanol was prepared and 1 mL of this solution was added to 3 mL of test solution in methanol at different concentrations. The absorbance was measured at 517 nm. A decrease in the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated in the equation using DPPH solution as control.

$$\%\text{DPPH scavenging activity} = [(\text{Control absorbance} - \text{Sample absorbance})/\text{Control absorbance}] \times 100$$

2.5. Acanthamoeba cultures

A. castellanii belonging to the T4 genotype, sourced from keratitis patient, were purchased from the American Type Culture Collection (ATCC 50492). The cultures were grown in 15 mL of peptone glucose yeast (PYG) medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] in T-75 tissue culture flasks at 37 °C without shaking [13]. The media were refreshed 15–20 h prior to experiments. *A. castellanii* adhering to flasks represented the trophozoite form and were collected by placing the flasks on ice for 30 min with gentle agitation and used in all experiments.

2.6. Amoebistatic and amoebicidal assays

Amoebistatic and amoebicidal assays were performed as previous described [16]. Briefly, *A. castellanii* were incubated with different concentrations of honey [10%, 20% and 30% (v/v) in PYG in 24-well plates (10^5 amoebae per 0.5 mL per well). Plates were incubated at 37 °C for 24 h. After this incubation, the number of amoebae was determined by haemocytometer counting. The counts from *A. castellanii* incubated with PYG alone were taken as 100% and effects of honey were presented as percent relative change. Glycerol (with similar viscosity) was used as control, using same concentrations as for honey *i.e.*, 10%, 20% and 30% (v/v), while sodium dodecyl sulphate (0.05%) was used to lyse 100% amoebae trophozoites.

Table 1
Natural and marketed honey samples used in the present study.

Sample no.	Honey type	Place of production
H1	Natural honey from bee hive	Collected directly from the bee hive from Rajanpur District of Southern Punjab
H2	Natural honey from bee hive	Collected directly from the bee hive from Rajanpur District of Southern Punjab
H3	Salman's honey (marketed sample)	Commercially produced in Pakistan
H4	Al Shifa honey (marketed sample)	Commercially produced in Saudi Arabia
H5	Young's honey (marketed sample)	Commercially produced in Pakistan

For amoebicidal assays, *A. castellanii* were incubated with different concentrations of honey [10%, 20% and 30% (v/v)] in phosphate buffer solution in 24-well plates (10^5 amoebae per 0.5 mL per well). Plates were incubated at 37 °C for 24 h. After this incubation, the number of amoebae was determined by haemocytometer counting. The counts from *A. castellanii* incubated with phosphate buffer solution alone were taken as 100% and effects of honey were presented as percent relative change. Glycerol and sodium dodecyl sulphate were used as controls.

Additionally, effects of natural honey and marketed honey on *A. castellanii* trophozoites were observed periodically under a phase contrast inverted microscope and representative images were recorded.

3. Result

3.1. Antiacanthamoebic activities of natural and marketed honey

Amoebistatic and amoebicidal properties of various concentrations of natural and marketed honey were determined. For amoebistatic assays, *A. castellanii* incubated with growth medium alone (PYG) for 24 h resulted in increase in numbers, from 10^5 amoebae to $2.8 \times 10^5 \pm 3.7 \times 10^4$ amoebae and this was considered as 100%. Natural honey exhibited significant amoebistatic effects in a concentration-dependent manner ($P < 0.01$ using two sample *t*-test; one-tailed distribution) (Figure 1A). At 10% honey, the number of *A. castellanii* was reduced to $6.8 \times 10^4 \pm 3.0 \times 10^3$

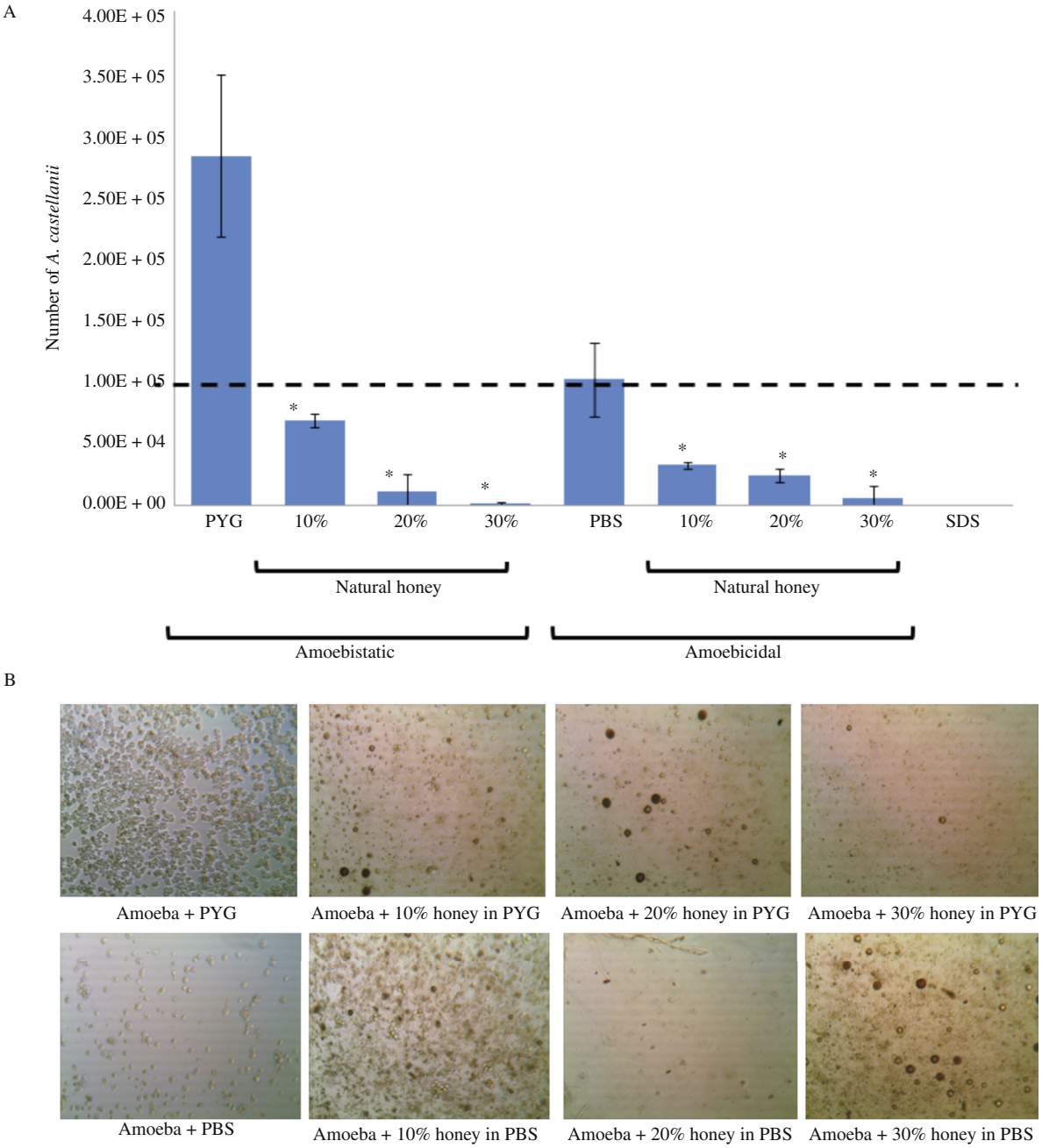

Figure 1. Amoebistatic and amoebicidal properties of natural honey.
A: Number of *A. castellanii* after treatment with natural honey. Both H1 and H2 showed similar effects, however only H1 data is shown. For amoebicidal effects, PYG was replaced with nutrient-free PBS. Again, natural honey exhibited significant amoebicidal effects at all concentrations tested ($P < 0.01$ using two sample *t*-test; one-tailed distribution); *:Significant difference; Data are presented as mean ± SE of three independent experiments performed in duplicate; B: Representative micrograph of *A. castellanii* incubated with and without natural honey (H1) (×100).

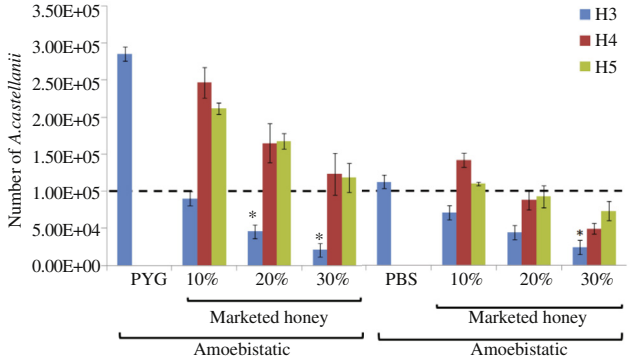

Figure 2. Amoebistatic and amoebicidal properties of marketed honey. *: Significant difference; Data are presented as mean ± SE of three independent experiments performed in duplicate.

as compared to the control ($2.8 \times 10^5 \pm 3.7 \times 10^4$), while 30% honey reduced amoebae number to $8.3 \times 10^2 \pm 8.3 \times 10^2$ as compared to the control ($2.8 \times 10^5 \pm 3.7 \times 10^4$). Consistent with these findings, natural honey exhibited significant amoebicidal effects in a concentration-dependent manner ($P < 0.01$ using two sample *t*-test; one-tailed distribution) as observed by reduction in amoebae numbers (Figure 1A). At 10% honey, the number of *A. castellanii* was reduced to $3.2 \times 10^4 \pm 1.45 \times 10^3$, while 30% honey reduced the number of *A. castellanii* to $5.8 \times 10^3 \pm 5.84 \times 10^3$ as compared to the control, *i.e.*, $1 \times 10^5 \pm 1.74 \times 10^4$ amoebae. When observed under the microscope, honey treated *A. castellanii* showed loss of acanthopodia initially, following which they detached, rounded up, reduced in size, decrease in cytoplasmic mass and were observed floating in the culture medium (Figure 1B). When treated with glycerol, amoebistatic and amoebicidal effects were observed, however, natural honey produced significantly higher amoebistatic and amoebicidal effects compared with glycerol ($P < 0.01$ using two sample *t*-test; one-tailed distribution). For amoebistatic effects, 30% honey reduced amoebae number to $8.3 \times 10^2 \pm 8.3 \times 10^2$, while 30% glycerol reduced amoebae number to $3.6 \times 10^4 \pm 1.7 \times 10^3$. For amoebicidal effects, 30% honey reduced amoebae number to $5.8 \times 10^3 \pm 5.84 \times 10^3$, while 30% glycerol reduced amoebae number to $5.4 \times 10^4 \pm 3.3 \times 10^3$. To determine whether honey and glycerol-treated amoebae remain viable, *A. castellanii* were inoculated in the growth medium, PYG, post-treatment with honey and glycerol. In honey-treated samples, no viable amoebae emerged within 24 h of incubation with PYG, however, glycerol-treated amoebae exhibited viable trophozoite and active growth (data not shown).

For amoebistatic assays, amoebae (10^5) were incubated with marketed honey samples (H3, H4, H5) for 24 h and enumerated. In growth medium (PYG) alone, amoebae number increased from original inoculum (dotted line) to $2.8 \times 10^5 \pm 3.7 \times 10^4$. Among marketed honey samples tested, H3 showed higher amoebistatic properties as compared to H4 and H5 (Figure 2). For amoebicidal effects, PYG was replaced with nutrient-free PBS. Consistently, amoebicidal effects of H3 sample (*i.e.*, $2.4 \times 10^4 \pm 7.3 \times 10^3$) were more pronounced compared with the amoebicidal effects of H4 ($4.9 \times 10^4 \pm 7.1 \times 10^3$) and H5 ($7.3 \times 10^4 \pm 1.3 \times 10^4$) ($P < 0.01$ using two sample *t*-test; one-tailed distribution). However, the amoebicidal effects of H4 and H5 were similar to the amoebicidal effects of glycerol ($5.4 \times 10^4 \pm 3.3 \times 10^3$). When inoculated in the growth medium, H3-, H4-, and H5-treated amoebae exhibited viable trophozoite and active growth (data not shown). Overall, the natural bee hive honey was more effective in inhibiting *A. castellanii* as compared to marketed honey.

3.2. Phenolic and flavonoid contents and antioxidant activities of natural and marketed honey

With potent antiamoebic effects of natural honey, we next determined phenolic and flavonoid contents and antioxidant activities of natural honey *vs.* marketed honey. The results (Figure 3A, B) revealed that among honey samples tested, the proportion of flavonoid and phenolic contents was found in the following order; H1 > H2 > H5 > H4 > H3, with an exception of slightly higher proportion of phenolic contents in H3 compared to its levels in H4. Natural honey showed higher flavonoid and phenolic contents compared with the marketed honey samples.

Natural honey showed higher antioxidant activities compared with the marketed honey samples (Figure 4A, B). As for flavonoid and phenolic contents, antioxidant activities were higher in natural honey samples compared with marketed honey samples. Notably, similar pattern of antioxidant activity was observed in both natural honey samples tested. Honey samples

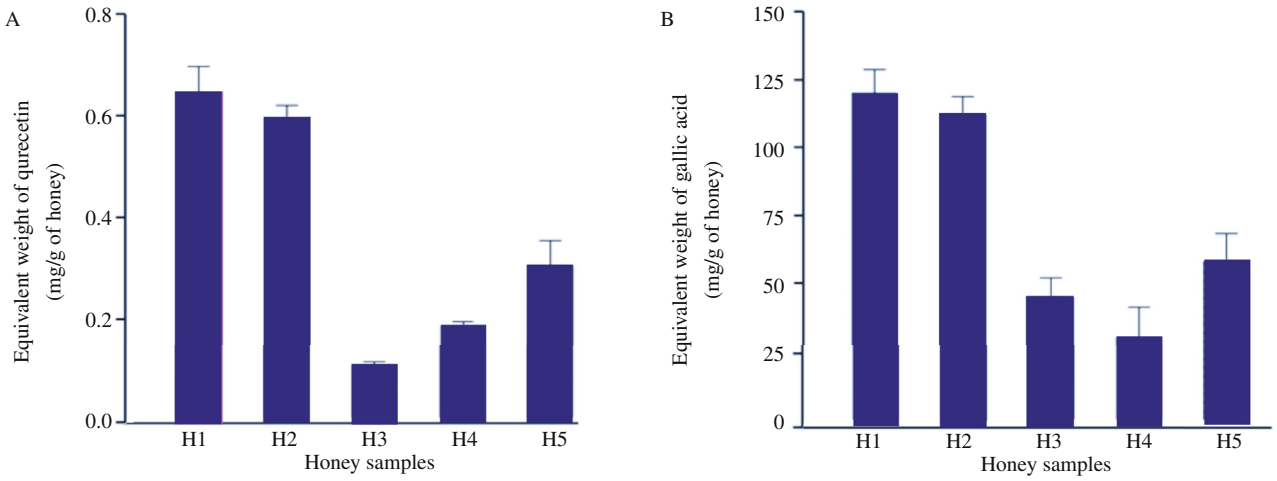

Figure 3. Flavonoid and phenolic contents of natural honey and market honey samples.
A: Flavonoid contents; B: Phenolic contents. Data are presented as mean ± SE of three independent experiments performed in duplicate.

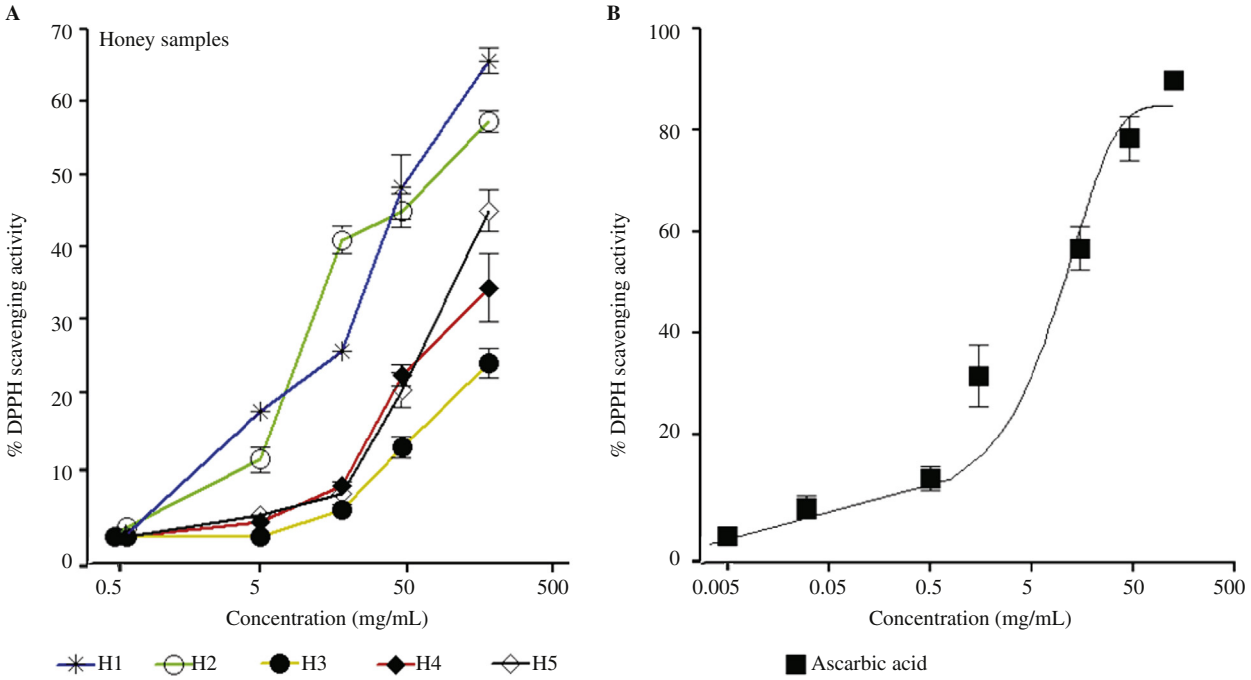

Figure 4. The antioxidant activities of natural honey (H1, H2) and marketed honey samples (H3, H4, H5) determined by measuring % DPPH scavenging activity.
Data are presented as the mean ± SE of three independent experiments performed in duplicate.

exhibited concentration-dependent % free radical scavenging activity with maximum effect at highest tested concentrations in the following order: H1 (65.66% ± 2.89%, n = 3)≥ H2 (57.33% ± 2.51%) > H5 (45% ± 5%) > H4 (34.33% ± 8.14%) > H3 (24.00% ± 3.46%).

4. Discussion

It is well established that honey is a natural product of medicinal value and widely used in communities for its wound healing, anti-inflammatory, and antibacterial properties [17,18]. The broad spectrum antibacterial properties of honey is multifactorial in nature, partly attributing to hydrogen peroxide, high osmolarity, antibacterial compound methylglyoxal [17], however, its source, production, manufacturing, and storage is likely to affect its contents and therapeutic properties. For example, Kwakman *et al.* [19], showed that Revamil medical-grade honey, produced under standardized conditions in greenhouses, has potent reproducible bactericidal activity suggesting that natural honey possess potent medicinal properties. Later, Kwakman *et al.* [17], identified defensin-1 as a potent antibacterial agent from honey, which is part of the honey bee immune system and is added by bees to honey.

Although antibacterial properties of natural honey have been well documented, there are no reports of effects of honey against pathogenic *Acanthamoeba* spp. For the first time, the present study showed that natural honey has antiacanthamoebic properties and possesses higher flavonoid, phenolic and antioxidant properties compared with the marketed honey. Phenolics and flavonoids are a group of bioactive low molecular weight compounds derived from plants and known for their antioxidant and anticancer properties. They occur as flavanones, flavones, flavonols, isoflavonoids, anthocyanins, and flavans. Flavonoids and phenolics exhibit health promoting effects such as reducing the risk of cancer, heart disease, asthma, stroke and brain tonic in relation to the antioxidant activity [20,21]. In the present study, natural honey exhibited potent amoebistatic and amoebicidal properties, compared with the marketed honey, albeit the molecular events of amoebae cytotoxicity require further studies. A comparison clearly indicates that the naturally sourced honey samples possess higher concentrations of flavonoids and phenolic with greater antioxidant potential than honey samples obtained from the local market. Thus the observed differences in antiamoebic properties may be attributed to variations in constituents of flavonoids and phenolic, or possibly a combination of other factors, however, the precise mechanisms are yet to be explored. It is also unclear whether the antiamoebic property of natural honey is due to an individual ingredient or a combination of antimicrobial components. By selectively neutralizing individual components present in natural honey, future studies will determine the underlying molecular mechanisms of antiamoebic properties of natural honey to identify novel antiamoebic factor(s). Such honeys, or isolated components thereof, could serve as novel agents to prevent or treat infections, in particular those caused by antibiotic-resistant bacteria, and serve as novel molecules to prevent or treat amoebic infections. A careful selection of honey, containing factors with antiamoebic and antibacterial properties would be of therapeutic value, in particular for topical use and/or may provide added benefit when supplemented with known chemical remedies for such infections. Additionally, the isolation of ingredients from natural honey should identify novel factors that could be of value against infections due to other pathogen free-living amoebae.

Overall, these findings suggest remarkable differences in antiamoebic of marketed *vs.* natural honey, and these differences are likely attributed to variations in constituents or properties of honey, including flavonoids, phenolic, defensing-1, osmolarity, pH, or possibly a combination of factors, however, the precise mechanisms are yet to be explored. These findings are of concern to the general public, health officials and to the local and

marketed honey manufacturers regarding the production and storage for standardization of honey for medical applications.

Conflict of interest statement

We declare that we have no conflict of interest.

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