Anti-acanthamoebic properties of natural and marketed honey in Pakistan

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Short title: Antiamoebic effects of honey

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Abstract

Objectives: To determine anti-Acanthamoebic 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 g of the extract) and phenolic content (gallic acid equivalent per g). 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 *A. 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 *A. 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 *versus* marketed honey. **Conclusions:** This study shows that natural honey has anti-Acanthamoebic 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.

Keywords: Honey; *Acanthamoeba*; Amoebicidal; Amoebistatic.

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 to 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], antitumor activities [8] 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 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*. *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 anti-Acanthamoebic activity of natural honey collected directly from the bee hive and compared its effects with the marketed honey samples, both of which are accessible to the local community. Antioxidant properties (polyphenols and flavonoids) of natural *versus* marketed honey were determined further.

2. Materials and methods

2.1.Source of honey samples

For natural honey, samples were collected directly from the bee hive from the Rajan Pur district of Southern Punjab, Pakistan. Two samples were collected from two different bee hives from Rajan Pur district. 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 (1g per mL) was added to an equal volume of 2% $AlCl_3.6H_2O$ 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 g 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 (1g per mL) and final volume adjusted to 46 mL by addition of distilled water. After 3 min, 3 mL of Na_2CO_3 (2%) 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 g of the test material.

2.4.Antioxidant activity using DPPH assay

The reducing power and free radical scavenging activity of test samples were determined using 2,2-diphenyl-1-picrylhydrazyl (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 centered 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 mg per mL) and the reference antioxidant, ascorbic acid (0.005 – 500 µg per mL) was dissolved in distilled water for free radical scavenging activity. A 0.1 mM 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.

$$\% \text{ DPPH scavenging activity} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100$$

2.5.*Acanthamoeba* cultures

Acanthamoeba 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 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^{o}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^{o}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 are presented as percent relative change. Glycerol (with similar viscosity) was used as a control, using same concentrations as for honey i.e., 10, 20 and 30% (v/v), while sodium dodecyl sulphate (SDS, 0.05%) was used to lyse 100% amoebae trophozoites.

For amoebicidal assays, *A. castellanii* were incubated with different concentrations of honey [10, 20 and 30% (v/v)] in PBS in 24 well plates (10^5 amoebae per 0.5 mL per well). Plates were incubated at 37^{o}C for 24 h. After this incubation, the number of amoebae was determined by haemocytometer counting. The counts from *A. castellanii* incubated with PBS alone were taken as 100% and effects of honey are presented as percent relative change. Glycerol and SDS was 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. Results

3.1. Anti-acanthamoebic 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 $2.8\times10^5 \pm 3.7\times10^4$ amoebae and this was considered as 100%. Natural honey exhibited significant amoebistatic effects in a concentration-dependent manner (P <0.01 using 2 sample T-test; one-tailed distribution) (Fig. 1A). At 10% honey, the number of *A. castellanii* was reduced to $6.8\times10^4 \pm 3.0\times10^3$ as compared to the control ($2.8\times10^5 \pm 3.7\times10^4$), while 30% honey reduced amoebae

number to $8.3\times10^2 \pm 8.3\times10^2$ as compared to the control ($2.8\times10^5 \pm 3.7\times10^4$). Consistent with these findings, natural honey exhibited significant amoebicidal effects in a concentration-dependent manner (P <0.01 using 2 sample T-test; one-tailed distribution) as observed by reduction in amoebae numbers (Fig 1A). At 10% honey, the number of *A. castellanii* was reduced to $3.2\times10^4 \pm 1.45 \times10^3$, while 30% honey reduced the number of *A. castellanii* to $5.8\times10^3 \pm 5.84\times10^3$ as compared to the control, i.e., $1\times10^5 \pm 1.74\times10^4$ 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 they observed floating in the culture medium (Fig. 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 2 sample T-test; one-tailed distribution). For amoebistatic effects, 30% honey reduced amoebae number to $8.3\times10^2 \pm 8.3\times10^2$, while 30% glycerol reduced amoebae number to $3.6\times10^4 \pm 1.7\times10^3$. For amoebicidal effects, 30% honey reduced amoebae number to $5.8\times10^3 \pm 5.84\times10^3$, while 30% glycerol reduced amoebae number to $5.4\times10^4 \pm 3.3\times10^3$. 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).

Among marketed honey, H3 showed higher amoebistatic properties as compared to H4 and H5 (Fig. 2). Consistently, amoebicidal effects of H3 sample (i.e., $2.4\times10^4 \pm 7.3\times10^3$) were more pronounced compared with the amoebicidal effects of H4 ($4.9\times10^4 \pm 7.1\times10^3$) and H5 ($7.3\text{x}10^4 \pm 1.3 \times10^4$). However, the amoebicidal effects of H4 and H5 were similar to the

amoebicidal effects of glycerol ($5.4\times10^4 \pm 3.3\times10^3$). 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 *versus* 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 [17, 18]. In the present study, the total flavonoid and phenolic contents estimated in natural honey samples tested (H1, H2) and marketed honey samples tested (H3, H4, H5) are expressed as mg of quercetin equivalent per g of the extract and mg of gallic acid equivalent/g of the extract, respectively (Fig. 3A and B). The results 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 H_3 compared to its levels in H_4.

The antioxidant activities of honey samples and the positive control of ascorbic acid are represented as % DPPH scavenging activity (Fig. 4A and B). Notably, similar pattern of antioxidant activity was observed in honey samples tested, as for flavonoid and phenolic

contents. Honey samples exhibited concentration-dependent % free radical scavenging activity with maximum effect (*E. max*) 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±3.46%) as shown in figure 3C. 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.

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 [19,20]. The broad spectrum antibacterial properties of honey is multifactorial in nature, partly attributing to hydrogen peroxide, high osmolarity, antibacterial compound methylglyoxal [19], however, its source, production, manufacturing, and storage is likely to affect its contents and therapeutic properties. For example, Kwakman et al., [21], 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., [19], 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 anti-Acanthamoebic properties and possesses higher flavonoid, phenolic and antioxidant properties compared with the marketed honey. Natural honey exhibited potent amoebistatic and amoebicidal properties, compared with the marketed honey, albeit the molecular events of amoebae cytotoxicity require further studies. It is also unclear whether the antiamoebic properties 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). It is hoped that such honeys, or isolated components thereof, may serve as novel agents to prevent or treat infections, in particular those caused by antibiotic-resistant bacteria. It is hoped that natural honeys, or isolated factor(s), could 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 *versus* 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.

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Figure legends

Figure 1. (A) Amoebistatic and amoebicidal properties of natural honey. For amoebistatic assays, amoebae (10^5) were incubated with natural honey for 24 h and enumerated. In growth medium (PYG) alone, amoebae number increased from original inoculum (dotted line) to $2.8\times10^5\pm3.7\times10^4$. Natural honey exhibited significant amoebistatic effects at all concentrations tested ($P<0.01$ using 2 sample T-test; one-tailed distribution). 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 2 sample T-test; one-tailed distribution). Asterisk (*) indicates significant difference. Data are presented as the mean ± standard error of three independent experiments performed in duplicate. **(B)** Representative micrograph of *A. castellanii* incubated with and without natural honey (H1) (x100).

Figure 2. Amoebistatic and amoebicidal properties of marketed honey. 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 $2.8\times10^5\pm3.7\times10^4$. Among marketed honey samples tested, only H3 exhibited potent amoebistatic effects at higher concentration. For amoebicidal effects, PYG was replaced with nutrient-free PBS. Again, only H3 honey sample, at 30% showed potent amoebicidal effects ($P<0.01$ using 2 sample T-test; one-tailed distribution). Asterisk (*) indicates significant difference. Data are presented as the mean ± standard error of three independent experiments performed in duplicate.

Figure 3. Natural honey showed higher flavonoid and phenolic contents compared with the marketed honey samples. The total flavonoid and phenolic contents in natural honey (H1, H2)

and marketed honey samples (H3, H4, H5) were determined by measuring quercetin and gallic acid. The proportion of flavonoid and phenolic contents was higher in natural honey samples compared with marketed honey samples. Notably, similar levels of flavonoid and phenolic contents were observed in both samples of natural honey. Data are presented as the mean ± standard error of three independent experiments performed in duplicate.

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