Original article

In vitro activity of some natural honeys against *Entamoeba histolytica* and *Giardia lamblia* trophozoites

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Seif Eldin A. Mohammed [a], Ahmed S. Kabbashi [b], Waleed S. Koko [b], Mohammad Javed Ansari [a], Nuru Adgaba [a], Ahmad Al-Ghamdi [a],*

[a] *Bee Research Chair, Department of Plant Protection, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia*
[b] *Medicinal and Aromatic Plants and Traditional Medicine Research Institute, P.O. Box 2404, National Center for Research, Khartoum, Sudan*

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ABSTRACT

Considering the potentiality of honey in combating diseases, the present study was carried out aiming to assess the in *vitro* antiprotozoal activity of several honeys (*Ziziphus spina-christi*, *Acacia nilotica*, *Acacia seyal*, and *Cucurbita maxima*) against *Entamoeba histolytica* and *Giardia lamblia* by employing the sub-culture method. All the tested honeys inhibited the growth of trophozoites, and the level of inhibition varied according to the assayed concentrations and incubation times. *Acacia seyal* honey had completely stopped motility of *E. histolytica* trophozoites at a concentration $\leq 50\ \mu g/ml$ after incubation for 72 h. *Ziziphus spina-christi*, *Acacia seyal*, and *Acacia nilotica* honeys had completely inhibited the growth of *Giardia lamblia* trophozoites at concentration $\leq 200\ \mu g/ml$ after 72 h. These inhibitory activities were similar to that of Metronidazole™ which showed IC_{50} = 0.27. The mammalian cytotoxicity of these honeys against normal Vero cell line which determined by applying MTT method verified the nontoxicity of the examined honeys. Also the proximate composition of the samples indicated compliance with the natural honey standards. The findings of the study indicate the need for *in vivo* studies and further investigations to identify active principles with antiprotozoal activities from natural honeys.

1. Introduction

The World Health Organization (WHO) estimates that the protozoan *Entamoeba histolytica* is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually (Ravdin and Stauffe, 2005). Intestinal amoebiasis due to the infection of *E. histolytica* is ranked as third on the list of parasitic protozoan infections leading to death behind malaria and schistosomiasis (Farthing et al., 1996). Amoebiasis is the infection of the human gastrointestinal tract by *E. histolytica*, a protozoan parasite capable of invading the intestinal mucosa and that may spread to other organs, mainly the liver which usually leads to an amoebic liver abscess (Samuel et al., 2001). Amoebiasis associated with dysentery is common in the less developed and developing countries of the world, especially in the tropics (Ravdin and Stauffe, 2005). For instance, 39% infections were reported in Bangladesh (Blessmann and Le Van Tanniech, 2006).

Giardiasis is the most common cause of parasitic gastrointestinal disease and it is estimated that up to two hundred million people are chronically infected with *Giardia lamblia* globally, and 500,000 new cases reported annually (Dahab et al., 2013). It causes significant gastrointestinal and diarrheal diseases in a wide variety of vertebrates including cats and human (Scorza et al., 2004; Lauwaet et al., 2010). It is one of the leading causative agents of diarrhea in both children (Addy et al., 2004; Noor Azian et al., 2007; Dib et al., 2008) and adults (Nyarango et al., 2008; Ayeh-Kumi et al., 2009). Metronidazole™ is the drug currently widely used and recommended in the treatment of amoebiasis and giardiasis (Towson et al., 1994). However; sometimes it causes adverse effects such as amyoplasia, neuralgia, and allergic dermatitis (Upcrof et al., 2006). These indicate the importance of seeking alternative treatments. Honey is a natural hive product with an extensive history of traditional human medicinal use, in a large number of societies. The use of honey in treating a wound and mild bacterial infection has long been known and practiced. Honey added to oral rehydration solution has been found to speed up

* Corresponding author.
E-mail address: aalkhazim1@gmail.com (A. Al-Ghamdi).

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recovery from vomiting and diarrhea in infants and children suffered from gastroenteritis (Abdulrhman et al., 2010).

The present work was initiated with the aim of screening some honeys for their *in vitro* antiprotozoal activities against *E. histolytica* and *G. lamblia* as well as to determine the composition and safety of different natural honeys.

2. Materials and methods

2.1. Collection of honey samples

Four different botanical origin honey samples were collected randomly from beekeepers during the season 2014. The collected samples were labeled from (1–4) and then stored at room temperature pending for analysis.

2.2. The proximate composition and pollen analysis

Proximate composition of the honey samples was determined using the methods of AOAC (1990; 2000). For moisture content, 2.0 g of each sample was dried to constant weight in hot air oven at 70 °C and the moisture was calculated on dry basis. Ash content was determined by drying 5.0 g of honey samples in crucibles at 105 °C for 3 h in oven. The dried samples were ignited in a furnace at 550–600 °C to constant weight, cooled and weighed. Protein content was determined using the micro-kjeldhal procedure to estimate the total nitrogen content and the protein content was calculated by multiplying the nitrogen by a factor ($\times 6.25$). Crude fat content was determined following extraction using Majonnier fat extraction apparatus. Carbohydrate contents of the honey samples were calculated as follows:

$$\%Carbohydrate = 100\% - (\%Moisture + \%Crude\ Fat + \%Crude\ Protein + \%Ash)$$

The energy values of the samples were calculated as follows:

$$Energy\ (KJ/100\ g) = 4.186\ [(\%Crude\ Protein \times 4) + (\%Crude\ Fat \times 9) + (\%Carbohydrate \times 4)]$$

The analysis of pollen of honey was carried out following standard acetolysis method of Erdtman (1969). Briefly, duplicate voucher slides were prepared from each honey. The pollen grains were stained with basic fuchsin and examined under the microscope using ($\times 400$). Pollen taxa were identified using pollen reference slides.

2.3. Parasites isolates

E. histolytica and *G. lamblia* used in the experiments were from clinical specimen patients of amoebiasis or giardiasis. All positive samples were examined by wet mount preparation. Then the positive sample was transported to the laboratory in nutrient broth medium. Trophozoites in the log phase were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% bovine serum at 37 ± 1 °C till used in the assays.

2.4. In vitro susceptibility assays

In vitro susceptibility assays used the sub- culture method (Cedillo-Rivera et al., 2002). Five mg from each honey sample were dissolved in 50 μl of dimethyl sulfoxide (DMSO) in eppendorf tube containing 950 μl distilled water in order to reach a concentration of 5 mg/ml. The concentrates were stored at -20 °C for further analysis.

Twenty μl of a honey solution of the serial dilutions of the honey samples added to each well of the sterile 96-well microplate in duplicate at final concentrations of 200, 100, 50, 25, 12.5 and 6.25 μg/ml. Metronidazole™ (a trichomonocide) [(1-(2-hydroxyethl)-2-methyl-5 nitroimidazole] was used as positive control, at concentrations of (312.5, 156.2, 78.1, 39.1, 19.5, and 9.7 μg/ml). 80 μl of culture medium was complemented with parasites and added to all wells. The final volume in the wells was 100 μl. Blank controls were included in each assay. Triplicate counts were performed with trypan blue by using hemocytometer after 24, 48, 72, 96, and 120 h. The mortality % of parasite for each honey sample was carried out according to the following formula:

$$\%\ Mortality\ of\ parasites = \frac{(Control\ negative - Test\ sample) \times 100\%}{Control\ negative}$$

Only 100% inhibition of the parasite considered when there was no motile parasite observed.

2.5. Cytotoxicity screening of honey

Mammalian cell cytotoxicity was evaluated using Vero cells following the method described by Patel et al. (2009). Briefly, 5 mg from each honey were weighed in eppendorf tubes. 50 μl of DMSO were added to the mixture and the volume was completed to 1 ml with distilled water. The mixture was vortexed and stirred by a magnetic stirrer. 20 μl of serial dilutions of each honey were added to each well of a 96 – well microplate in three duplicated concentrations (500, 250, 125 μg/ml). Triton-X was used as positive control and a blank control was included in each assay. 80 μl of complete medium were added to all used wells. 100 μl of cell suspension were added completing all wells to the volume 200 μl. Then the plates were covered and incubated at 37 °C for 96 h. To each well of the 96-well plate, 50 μl of diluted (1:3.5) MTT [5 mg/ml in 100 ml phosphate buffer solution] were added. The plate was incubated for a further 4 h at 37 °C. MTT was removed, and 100 μl of DMSO were added to each well. The plate was agitated at room temperature for 10 min then read at 540 nm using microplate reader.

The percentage growth inhibition was calculated using the formula below:

$$\%\ cell\ inhibition = 100 - \{(Ac - At)/Ac\} \times 100$$

where: At = Absorbance value of test compound; Ac = Absorbance value of the control.

3. Results

3.1. In vitro activity of honey against Entamoeba histolytica

All honeys examined against *E. histolytica* have shown varying *in vitro* inhibitory activity on parasite growth (Figs. 1–4). The level of inhibition varied according to the assayed concentrations and incubation periods. In all cultures, the number of living trophozoites was significantly lower than non-treated cultures. The positive control (metronidazole™-treated) cultures showed a remarkable decrease in the parasite growth giving *IC50* value of 0.27 (Table 1).

In assays with different honeys, higher reduction in parasite growth ≥ 60% was observed in cultures exposed to all assay concentrations. The initial screening for the antiamoebic activity of the honeys showed that *Acacia seyal* honey had completely stopped motility of *E. histolytica* at a concentration ≤ 50 μg/ml after incubation for 72 h (Fig. 1). *Ziziphus spina-christi* and *Acacia nilotica* honeys had completely stopped motility of *E. histolytica* at a concentration ≤ 200 μg/ml after incubation for 72 h shown,
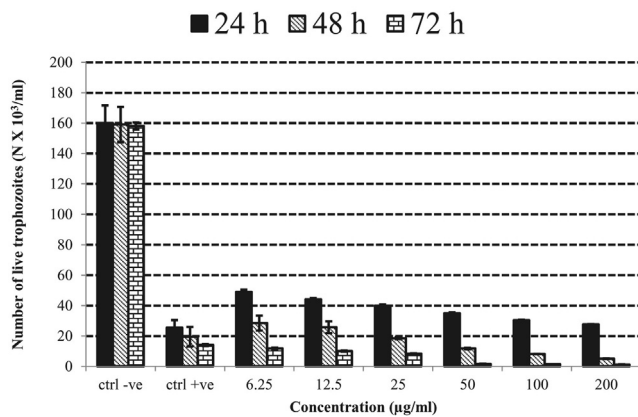
Fig. 1. *In vitro* effects of different *A. seyal* honey concentrations (microgram per milliliter) on the *E. histolytica* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.
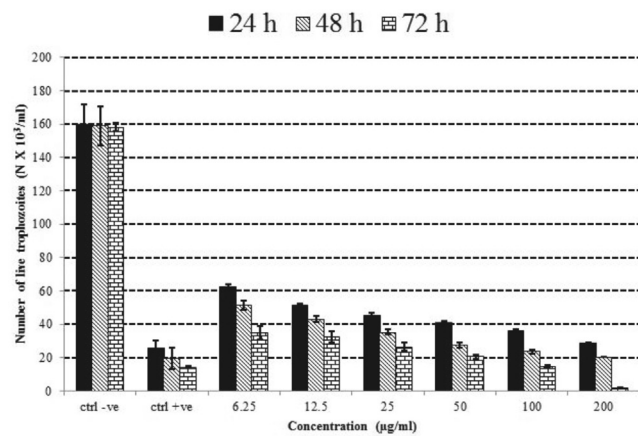

Fig. 2. *In vitro* effects of different *Ziziphus spina-christa* honey concentrations (microgram per milliliter) on the *E. histolytica* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.

Fig. 3. *In vitro* effects of different *A. nilotica* honey concentrations (microgram per milliliter) on the *E. histolytica* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.
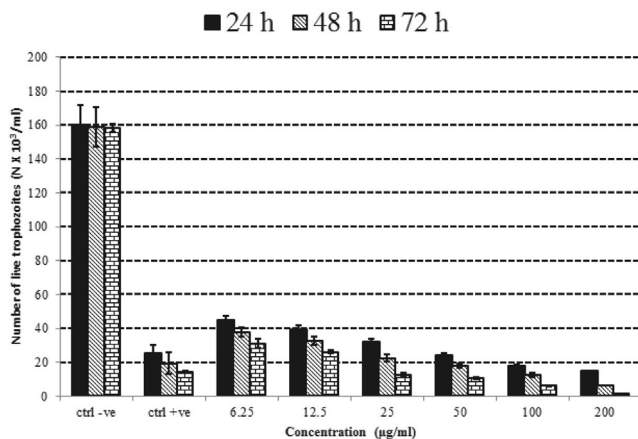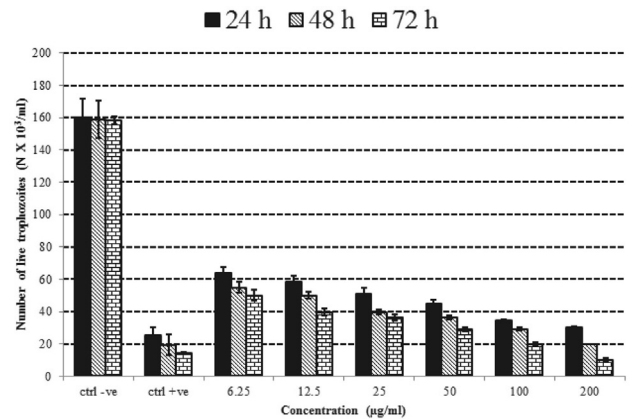

Fig. 4. *In vitro* effects of different *Cucurbita maxima* honey concentrations (microgram per milliliter) on the *E. histolytica* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.

Table 1
In vitro antiprotozoal activities of the honey samples.

No.	Test substance	IC$_{50}$ (µg/ml)	
		Antiamoebic	Antigiardial
1	*Ziziphus spina-christi* honey	0.68 ± 0.07	0.68 ± 0.02
2	*Acacia nilotca* honey	0.79 ± 0.07	1.20 ± 0.02
3	*Acacia seyal* honey	0.40 ± 0.08	0.75 ± 0.07
4	*Cucurbita maxima* honey	0.93 ± 0.05	8.07 ± 0.66
5	Metronidazole™	0.27 ± 0.01	0.27 ± 0.01

Data presented are mean IC$_{50}$ values of triplicate readings (µg/ml) ± SD.

respectively in Figs. 2 & 3. As shown in Fig. 4 no complete trophozoites motility was observed at this time point with *Cucurbita maxima* honey.

3.2. In vitro activity of honey against Giardia lamblia

Metronidazole-treated cultures showed time dependent inhibition of the parasitic growth. A significant ≥ 90% *G. lamblia* trophozoites growth inhibition was observed after 42, 48, and 72 h (Figs. 5–8) with IC_{50} = 0.27 (Table 1).
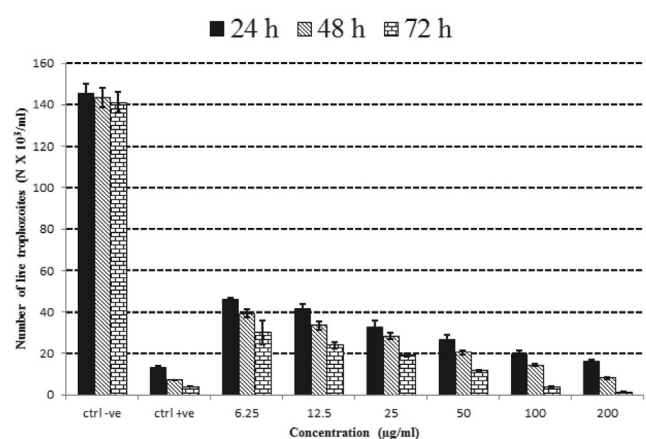

Fig. 5. *In vitro* effects of different *Ziziphus spina-christa* honey concentrations (microgram per milliliter) on the *G. lamblia* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.
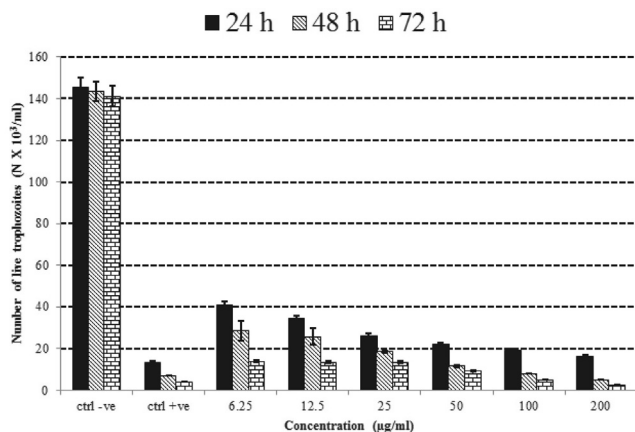
Fig. 6. *In vitro* effects of different *A. seyal* honey concentrations (microgram per milliliter) on the *G. lamblia* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.
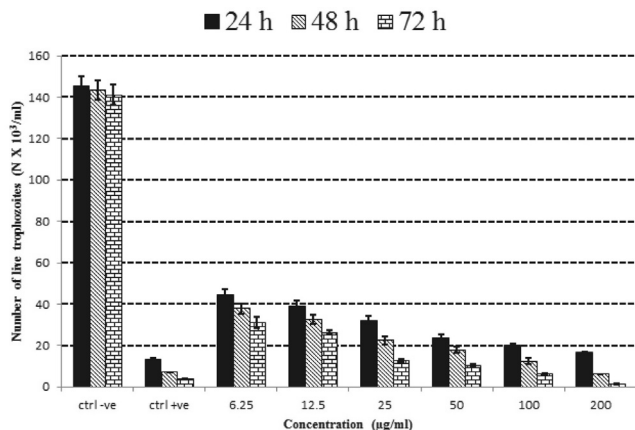

Fig. 7. *In vitro* effects of different *A. nilotica* honey concentrations (microgram per milliliter) on the *G. lamblia* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.
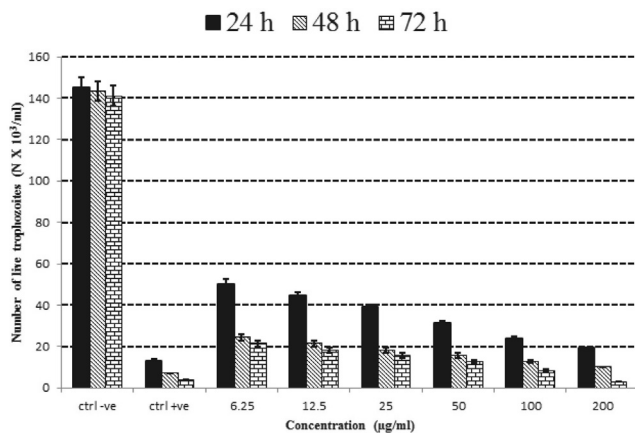

Fig. 8. *In vitro* effects of different *Cucurbita maxima* honey concentrations (microgram per milliliter) on the *G. lamblia* trophozoites after incubation for 24, 48, and 72. Negative (untreated cultures) and positive (metronidazole-treated cultures) controls were included in all assays. Data expressed as means of trophozoites' number ($\times 10^3$) ± SD of three similar assays.

Ziziphus spina-christi, *Acacia seyal*, and *Acacia nilotica* honeys had significantly reduced the numbers of motile *G. lamblia* trophozoites, results are consecutively depicted in Figs. 5–7. The growth inhibition of the three types of honey was observed with exposure to 6.25, 12.5, 25, 50 µg/ml honeys after incubation for 24 and 48 h till complete inhibition of growth achieved at concentration $\leq$ 200 µg/ml after 72 h. As depicted in Fig. 8, no complete trophozoites growth inhibition was observed at this time point with *Cucurbita maxima* honey.

3.3. Proximate composition, palynology, and cytotoxicity of honey

The pollen mono-morphs of the studied honey samples and the identified pollen are given in Fig. 9 and Table 3. The cytotoxicity of honey against Vero cell line is presented in Table 2. No cytotoxicity for the studied honeys was observed at assayed concentrations up to 500 µg/ml. Results of the proximate composition of the different honey samples are presented in Table 3. Only ash contents differed considerably among the studied samples.

4. Discussion

In the present study, all honeys tested against *E. histolytica* have shown varying *in vitro* trophozoiticidal activity. These results coincide with similar studies performed by other authors on the antiprotozoal activity of natural products (Khan et al., 2000; Shinohara et al., 2006; Almeida et al., 2007; Li et al., 2012).

Ziziphus spina-christi, *Acacia seyal*, and *Acacia nilotica* honeys had completely inhibited the growth of *G. lamblia* trophozoites at concentration $\leq$ 200 µg/ml after 72 h. These results agree with findings of Al-masoudi (2011) who studied anti-trophozoite activity of honey mixed with *zingiber officinale*, he obtained 97% mortality of trophozoites. Other studies also reported the successful use of honey and plants as antiparasitic agents. Freitas et al. (2006) studied *in vitro* activity of propolis crude extract against *Giardia duodenalis*. They observed 60% trophozoite growth inhibition by using 250–500 µg/ml concentrations. Damiani et al. (2010) reported 60.5–90% mortality of *varroa destructor* "ectoparasitic mite of honeybee colonies" using different propolis samples from Argentina. Previous investigation indicated that antiprotozoal property of honey as due to some bioactive glycoproteins and glycopeptides (Mohammed et al., 2015). Other reports have attributed the antiparastic activity of honey due to minor bioactive components rather than the major sugar constituents of honey. Sajid and Azim (2012) reported the anti-*caenorhabditis elegans* activity of honey as due to 2–10 kDa bioactive protein fractions. Also, it is reported that human & bovine milk contains substances such as milk apo-lactoferrin, casein, and secretory immunoglobulin type A which are responsible to the antiparasitic actitivity (León-Sicairos et al., 2006).

The physicochemical properties of some honeys, as well as their biological activities, are well covered by many investigators (Farouk et al., 1988; Mohammed and Ali, 2005; Mohammed and Babiker, 2009; Mohammed and Azim, 2012). Nevertheless, there is research gap considering the antiparasitic activity of these honeys. The proximate composition of honey in the present study, revealed no considerable variation. Fortunately, the cytotoxicity test of the honey samples against Vero cell line proved no cytotoxicity for the studies honeys at assayed concentrations up to 500 µg/ml. in addition to, the pollen mono-morphs of the studied honey samples corresponded with the botanical origin labeled out in each honey sample.

Protozoal diseases constitute major health problems worldwide, particularly in tropical developing countries. Among the protozoal parasites, *G. lamblia* and *E. histolytica* have the highest

Fig. 9. Identified pollen mono-morphs from the honey samples.

Table 2
Cytotoxicity of the honey samples.

No.	Test substance	Concentration (µg/ml)			The degree of toxicity
		% Inhibition[a]			
		500	250	125	
1	*Ziziphus spina-christi* honey	21.1 ± 0.05	12.8 ± 0.03	−10 ± 0.02	NT[b]
2	*Acacia nilotca* honey	17.0 ± 0.04	10.8 ± 0.02	−24.0 ± 0.03	NT[b]
3	*Acacia seyal* honey	21.1 ± 0.05	8.0 ± 0.01	−23.9 ± 0.02	NT[b]
4	*Cucurbita maxima* honey	16.0 ± 0.03	4.9 ± 0.02	−28.0 ± 0.01	NT[b]
5	MTT[d]	95.3 ± 0.00			HT[c]

[a] Data presented are mean inhibition (%) ± SD., n = 3.
[b] NT = not toxic (IC_{50} < 100 µg/ml).
[c] HT = highly toxic (IC_{50} > 30 µg/ml).
[d] MTT = Triton-x100 [(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was used as positive control at 0.2 µg/mL. The maximum concentration used was 500 µg/mL.

Table 3
Pollen analysis and proximate composition of the honey samples.

Parameters	Family	Honey sample code			
		1	2	3	4
		Rhamnaceae	Fabaceae	Fabaceae	Cucurbitaceae
	Pollen type	*Ziziphus spina-christi*	*Acacia nilotca*	*Acacia seyal*	*Cucurbita maxima*
% Moisture	16.07	17.04	16.38	17.37	
% Ash	0.26	1.23	1.44	0.12	
% Protein	1.14	1.75	1.75	0.87	
% Fiber	–	–	–	–	
% Carbohydrate	82.39	79.66	80.13	81.42	
Energy (kJ/100 g)	1403.9	1375.26	1382.30	1386.15	

incidence of diarrheal diseases in developing countries. Chemotherapy is the first choice for the treatment of protozoal diseases; however, it has proven side effects. Therefore, there is a need for nontoxic and effective treatment alternatives including honey as one of the readily available alternatives for control and prevention of protozoa. In a conclusion, the present study revealed that natural honey has the potential to inhibit protozoan growth. Hence, routine consumption of honey as prophylactic could help healing from diarrhea. However, further investigations on the honeys regarding the mode of action and other related pharmacological studies such as *in vivo* investigation, drug formulation, and clinical trials are needed.

Declaration of interest

The authors confirm that there is no conflict of interests and are also liable for the content and writing of this article.

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