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Original Article

Honey inhibits the *in vitro* growth of four *Babesia* species and *Theileria equi*

Mahmoud R. AbouLaila[1*], Soad M. Menshawy[1], Mohmed A. Rizk[3] and Ikuo Igarashi[2]

[1]Department of Pathology and Parasitology, Faculty of Veterinary Medicine, Damanhour University, Damanhour 22511, El-Behera, Egypt

[2]Department of Disease Control, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-Cho, Obihiro, Hokkaido 080-8555, Japan

[3]Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt

*Corresponding author: Dr. Mahmoud AbouLaila

E-mail: hethet2004@yahoo.com

Abstract:

Honey has antioxidant, immunostimulant, antibacterial, and antileishmanial activities. In this study, we evaluated the *in vitro* babesicidal and theilericidal effects of honey on *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, *Babesia caballi*, and *Theileria equi.* There was noteworthy suppression of growth at a concentration of 0.5% (V/V) for *B. bovis*, *B. bigemina*, *B. divergens*, and *T. equi* and 1% (V/V) for *B. caballi*. The IC_{50} values of honey were 1.98, 1.82, 0.42, 1.7, and 1.43% (V/V) for *B. bovis*, *B. bigemina*, *B. divergens*, *B. caballi*, and *T. equi*, respectively. The growth was entirely repressed at 1% (V/V) for *B. divergens*, 2.5% (V/V) for *T. equi*, and 5% (V/V) for *B. bovis*, *B. bigemina*, and *B. caballi*. The regrowth was repressed in the viability test at a concentration of 1% (V/V) for *B. divergens*, 2.5% (V/V) for *T. equi*, and 5% (V/V) for *B. caballi*, *B. bovis*, and *B. bigemina.* These results indicate honey as a natural killer of *Babesia* species and *T. equi.* Its use in the treatment of clinical cases requires further *in vivo* evaluation.

Key words: Honey - *Babesia* - *Theileria equi* - *In vitro*

INTRODUCTION

Blood parasites of the *Babesia* and *Theileria* species are the major cause of piroplasmosis in bovines and equines. They are spread by Ixodid ticks. *Babesia bovis*, *Babesia bigemina*, and *Babesia divergens* infect cattle and cause high economic losses in the livestock industry **(*Uilenberg, 2006)***.

Babesia caballi and *Theileria equi* infect equines and affect animal trade throughout the world. The infection is marked by fever, hemolytic anemia, icterus, hemoglobinuria, and death in some cases **(*Homer et al., 2000*)**. Several chemotherapeutic drugs, such as diminazene aceturate and imidocarb dipropionate, are used for the remedy of the disease, but they have toxic side effects ***(Vial and Gorenflot, 2006)*** that need to be eliminated. Therefore, it is vital to search for new drugs without toxic side effects.

Natural products have been assessed as antibabesial *in vitro* and *in vivo* in a rodent model **(*AbouLaila et al., 2018; AbouLaila et al., 2010; Salama et al., 2014*)**. Honey is a natural product that might be suitable for evaluation as an antibabesial agent. Honey

has been famous through history for its curative effects on several disease conditions in several civilizations. It contains many naturally occurring components, such as flavonoids, phenolic compounds, vitamins, trace elements, amino acids, and proteins **(*Alvarez-Suarez et al., 2010*)**, along with certain enzymes, including glucose oxidase, invertase, and catalase **(*Bogdanov et al., 2008; Doner, 1977; Weston, 2000*)**. Honey has antioxidant **(*Zoheir et al., 2015*)**, antiproliferative and anticancer **(*Attia et al., 2008; Catchpole et al., 2015; Jaganathan and Mandal, 2009*)**, anti-inflammatory **(*Al-Waili, 2003; Ansorge et al., 2003*)**, antidiabetic **(*Arabmoazzen et al., 2015*)**, antiviral **(*Zeina et al., 1996*)**, antibacterial **(*Asadi-Pooya et al., 2003; Basson and Grobler, 2008; Bastos et al., 2008; Boateng and Diunase, 2015*)**, antifungal **(*Koc et al., 2009*)**, antischistosomal **(*Mostafa, 2005; Mostafa and Soliman, 2010)**, anti-amoebic **(*WAW and Alvieno, 2012)**, antileishmanial **(*Bassam et al., 1997; Falcão et al., 2014; Fattahi Bafghi et al., 2007; Kaewmuangmoon et al., 2012; Nilforoushzadeh et al., 2010; Nilforoushzadeh et al., 2007; Wadi et al., 2015*)**, antitrypanosomal **(*Falcão et al., 2014)**, and antimalarial **(*Falcão et al., 2014; Kaewmuangmoon et al., 2012)*** properties.

The goal of the study was to assess the *in vitro* inhibitory effect of honey on four *Babesia* species and *T. equi*.

MATERIALS AND METHODS

1. Chemicals:

Honey (Pure honey) was purchased from a market in Obihiro, Japan (Shoei Co. Ltd., Japan). Honey is collected by honeybees in the primeval springtime from various flowers such as the flower of rapeseed, rape blossom, and mountain flower. It was dissolved in autoclaved double-distilled water to create a stock solution of 20 % (V/V) and filtered using a 0.22 µm syringe filter (Millipore, USA). The filtered solution was immediately mixed with a culture medium. SYBR Green I (SGI) nucleic acid stain (Lonza, USA; 10,000x) was kept at -20°C and thawed before use **(*Rizk et al., 2015)***. A lysis buffer comprising Tris (130 mM; pH 7.5), Ethylenediaminetetraacetic acid (EDTA) (10 mM), saponin (0.016%; W/V), and TritonX-100 (1.6%; V/V) was prepared earlier and stored at 4°C **(*Rizk et al., 2015*)**. A diminazene aceturate (Ciba Gigi Ltd., Japan) stock solution of 56 mg/ml was arranged and kept until use.

2. Parasites:

The parasites were *B. bovis* (Texas strain) **(*Bork et al., 2004)***, *B. bigemina* (Argentina strain) **(*Igarashi et al., 1998)***, *B. divergens* (German strain) **(*Rizk et al., 2016)***, and USDA strains of *B. caballi* **(*Bork et al., 2004*)** and *T. equi* **(*Mehlhorn and Schein, 1998*)**.

3. *In vitro* culture of the parasites:

Parasites were cultivated in cow or horse red blood cells using a continuous micro-aerophilous stationary phase culture system **(*Bork et al., 2004*)**. The culture medium M199 (Sigma-Aldrich) was used for *B. bovis*, *B. bigemina*, and *T. equi* and was appended with 40% cow or horse serum and 60 U/ml of penicillin G, 60 µg/ml of streptomycin, and 0.15 µg/ml of amphotericin B (Sigma-Aldrich). Hypoxanthine from ICN Biomedicals, Inc.(Aurora, OH, USA) was added to the *T. equi* culture as an energetic enhancement at 13.6 mg/ml. The RPMI 1640 medium was augmented with antibiotics,

amphotericin B, and either 40% horse serum for *B. caballi* **(*AbouLaila et al., 2010*)** or 10 % calf serum for *B. divergens* **(*AbouLaila et al., 2017)***.

4. *In vitro* growth inhibition assay:

The *in vitro* inhibition assay for honey was lead utilizing a fluorescence-based assay as previously described **(*Rizk et al., 2016; Rizk et al., 2015)***. Cow and horse RBCs at 1% parasitemia were additional to the culture at HCT values of 2.5% for *B. bovis* and *B. bigemina* and 5% for *B. caballi*, *B. divergens*, and *T. equi* packed RBCs inoculum **(*Rizk et al., 2015)***. The total volume in each well was 100 µl. Honey was used at concentrations of 0.01, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10% (V/V) of the culture medium (equal to 0.0143, 0.143, 0.35, 0.71, 1.43, 3.6, 7.15, and 14.3 mg/ml, respectively). The change from milliliter to milligram was conducted using a honey-amount converter (http://convert-to.com/246/honey-amounts-converter.html). Negative controls without drug-containing either fresh or infected RBCs at the same HTC value were included. Diminazene aceturate was used at concentrations of 0.0001, 0.0005, 0.001, 0.01, 0.1, 0.25, 0.5, 1, 1.5, 3.5, 7, and 14 mg/ml. Double distilled water (DDW) control plates were prepared for *B. bovis* and *B. caballi* using bovine and equine RBCs at the same HTC values and concentrations of honey in the drug experiment to determine any effect of the solvent on parasite growth. The plates were incubated for four days lacking shifting media at 37 ° C in an atmosphere containing 90 % N_2, 5 % CO_2, and 5 % O_2 in a humidified multi-gas water-jacketed incubator. A 2× SGI (10,000x) nucleic acid stain was mixed with 100 µl of a lysis buffer and added directly to each dilution by light mixing (Rizk *et al*., 2015), then stored for 6 hours at room temperature in a dark place. A fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific, USA) was utilized to determine the fluorescence values at 485 nm (excitation) and 518 nm (emission) wavelengths. The experiments were conducted thrice in triplicate. The parasitemia levels were deliberate after deletion of the RBC background **(*Rizk et al., 2015)***. The values were used to make a regression curve to obtain IC_{50} values.

5. Viability test:

Plates were prepared as for the *in vitro* inhibition assay and incubated for four days with the same media. The media were uninvolved, and infected erythrocytes were moved to a novel plate containing 100 µl of the culture medium alone. The percentage of infected and fresh RBCs were 42.8 and 57.2 % of the total RBC concentration, respectively. Plates were incubated for five days with the same media **(*AbouLaila et al., 2018*)**.

6. Statistical analysis:

JMP software (SAS Inc., USA) was utilized to detect significant values among different concentrations and the control using a student's *t*-test ($P < 0.05$).

RESULTS

Honey significantly inhibited growth at a concentration of 0.5% (V/V) for *B. bovis*, *B. bigemina*, *B. divergens*, and *T. equi* and 1% (V/V) for *B. caballi*. The growth was completely inhibited at 1% (V/V) for *B. divergens*, 2.5% (V/V) for *T. equi*, and 5% (V/V) for *B. bovis*, *B. bigemina*, and *B. caballi*. The IC_{50} values of honey were 0.42, 1.98, 1.82, 1.7, and 1.43% (V/V) (equal to 600, 2850, 2620, 2440, and 2100 µg/ml) for *B. divergens*, *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*, respectively **(Table 1)**. The IC_{50} values of diminazene aceturate were 0.4, 0.011, 0.19, 0.25, and 0.12 µg/ml for *T. equi, B. caballi*, *B. divergens*, *B. bovis*, *B. bigemina*, and, separately. *B. caballi* and

B. bovis growth in all the concentrations of DDW control was similar to the infected RBC negative control growth (not shown). Regrowth was inhibited in the viability test at a concentration of 1% (V/V) for *B. divergens*, 2.5% (V/V) for *T. equi*, and 5% (V/V) for *B. caballi*, *B. bovis*, and *B. bigemina* **(Table 2)**.

DISCUSSION

Honey inhibited the *in vitro* growth of four *Babesia* species and *T. equi. B. divergens* was the most sensitive to honey, while *B. bovis* was the least sensitive. The solvent had no inhibitory effect on the parasites; therefore, the inhibition was due to the honey. The IC_{50} values of honey for *Babesia* and *T. equi* were very high compared with those for diminazene aceturate in this study. The IC_{50} values of honey for *Babesia* and *T. equi* were similar to the IC_{50} values for *Caenorhabditis elegans* (0.83%) **(*Azim and Sajid, 2009*)**. The IC_{50} values of honey for *Babesia* and *T. equi* were higher than those for *Plasmodium falciparum* (30.1 µg/ml) **(*Falcão et al., 2014; Kaewmuangmoon et al., 2012*)**, *Leishmania* species (229.3 µg/ml) **(*Machado et al., 2007)***, *Trypanosoma brucei* (8.6 µg/ml), and *T. cruzi* (5.7 µg/ml) **(*Falcão et al., 2014*)**. This might be due to the use of honey extracts in the inhibition experiments of other parasites.

The IC_{50} values of honey for *Babesia* and *T. equi* were lower than those for the tumor cell lines of MCF-7, MDA-MB-231, and HeLa (10, 5, and 5 % (V/V), respectively) **(*Fauzi et al., 2011*)**. Moreover, concentrations of 1–10 % (V/V) had no outcome on the normal breast epithelial cell line, MCF-10A **(*Fauzi et al., 2011)***. Therefore, honey is safe for the remedy of *Babesia* and *T. equi* infections.

The effective mode of honey against tumors includes the regulation of the cell cycle, stimulation of the mitochondrial pathway, disruption of the outer mitochondrial membrane, initiation of apoptosis, modulation of oxidative stress, amendment of inflammation, the inflection of insulin signaling, and inhibition of *angiogenesis* **(*Erejuwa et al., 2014*)**. The action of honey is mainly the result of its high phenolic compound content **(*Kassim et al., 2010a; Kassim et al., 2010b*)**. Additional studies are desired to explain its action on *Babesia* and *Theileria* parasites.

In summary, honey inhibited the *in vitro* growth of *T. equi* and *Babesia* species. Moreover, it withdrew the growth in the viability test. While it has *in vivo* immunostimulant, anticancer, and antischistosomal properties, further studies are required to evaluate its *in vivo* inhibitory effect on *Babesia* and *Theileria* parasites.

Table (1): IC_{50} values of honey for *B. divergens*, *B. bovis*, *B. bigemina*, *B. caballi*, and *T. equi*

	IC_{50}s in µg/ml (=V/V)[a]	
Organism	**Honey**	**Diminazene aceturate**
B. divergens	600 (0.42)	0.19
B. bovis	2850 (1.98)	0.25
B. bigemina	2620 (1.82)	0.12
B. caballi	2440 (1.7)	0.011
T. equi	2100 (1.43)	0.4
P. falciparum [b]	4.4-30.1 (0.003-0.02)	ND
Leishmania species [c]	2.8- 229.3 (0.001-0.16)	ND
Trypanosoma brucei [d]	6.2 -8.6 (0.004- 0.006)	ND
T. cruzi [d]	1.7-5.7 (0.0012-0.003)	ND
MCF-7 tumor cell line [e]	14285.7 (10)	ND
MCF-10A normal breast cells [e]	NE	ND

[a] IC_{50} values are in microgram-per-milliliter concentrations of the culture medium for experiments carried out for three times in triplicate. Related IC_{50}s in percent volume/volume (V/V) are in parentheses.
[b] ***Kaewmuangmoon et al. 2012***. [c] ***Machado et al. 2007***. [d] ***Falcão et al. 2014***. [e] ***Fauzi et al. 2011***.
ND: not determined. NE: no effect at 14285.7 µg/ml (10% V/V).

Table (2): Viability of *Babesia* species and *T. equi* after 5 days of honey treatment

	Concentration (% V/V)							
Parasite	**0.01**	**0.1**	**0.25**	**0.5**	**1**	**2.5**	**5**	**10**
B. divergens	+	+	+	+	—	—	—	—
B. bovis	+	+	+	+	+	+	—	—
B. bigemina	+	+	+	+	+	+	—	—
B. caballi	+	+	+	+	+	+	—	—
T. equi	+	+	+	+	+	—	—	—

(+) viable and (—) dead

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الملخص العربي

مدى تأثير عسل النحل على نمو طفيليات البابيزيا و الثيليريا إكوي المزروعه معمليا

محمود رزق أبوليلة [1] و سعاد محمد منشاوي[1] و محمد عبده رزق[3] و إكيوإجراشي [2]

[1] قسم الباثولوجيا و الطفيليات كلية الطب البيطري جامعة دمنهور-مصر و[2]قسم السيطره على الأمراض-المركزالقومي لأمراض البروتوزوا جامعة أوبيهيرو-اليابان و[3]قسم الأمراض الباطنه و المعديه كلية الطب البيطري جامعة المنصوره

لعسل النحل فؤائد عديده كمضاد للأكسده ومنشط للمناعه و مضاد للبكتريا و مضاد لليشمانيا. تم في هذه الدراسه تجربة العسل كمضاد للبابيزيا و الثيليريا على البابيزيا بوفيز و البابيزيا بيجيمينا و البابيزيا دايفيرجينز و البابيزيا كابلي و الثيليريا إكوي معمليا. و كان للعسل تأثير معنويا مانع للنمو عند تركيز 0.5 % لكل من البابيزيا بوفيز و البابيزيا بيجيمينا و البابيزيا دايفيرجينز و الثيليريا إكوي و 1% للبابيزيل كابالي. وكان التركيز القاتل ل50% من الطفيليات هو 1.98 و 1.92و 0.42 و 1.7 و 1.43 % لكل من البابيزيا بوفيز و البابيزيا بيجيمينا و البابيزيا دايفيرجينز و البابيزيا كابلي و الثيليريا إكوي على الترتيب. لم تنمو الطفيليات ثانية في إختبار إعادة النمو عند تركيز 1% للبابيزيا دايفيرجينز و 2.5% للثيليريا إكوي و 5% لكل من البابيزيا بوفيز و البابيزيا بيجيمينا و البابيزيا كابلي. أوضحت نتائج الدراسه ان عسل النحل قاتل طبيعي لطفيليات البابيزيا و الثيليريا إكوي و يجب عمل دراسات على الحيوانات لمعرفة مدى إمكانية إستخدامه كعلاج في الحالات الإكلينيكيه.