# *In Vitro* Study on Anti-Amoebic Activity of Tualang Honey against *Entamoeba histolytica* Trophozoite

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Published 1 July 2012

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**ABSTRACT:** Anti-bacterial properties of Malaysian honeys have been widely studied. However, the anti-amoebic activity of local honeys against *Entamoeba histolytica* is still not known. This preliminary study was conducted to evaluate the *in vitro* activity of Tualang honey on the growth of *E. histolytica* trophozoites. Anti-amoebic activity of Tualang honey was assessed using a broth dilution method with metronidazole as the reference. Different concentration of honey [6.25% – 25% (weight/volume)] and metronidazole (0.78 – 100 µg/ml) were tested against HM-1:IMSS strain *E.histolytica* trophozoites in 96-well flat-bottom microtiter plate under anaerobic condition. The growth of trophozoites in each well was evaluated microscopically using inverted microscope. Trophozoites were monitored for motility and morphological changes (rounding-up) and the numbers were scored accordingly. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of honey dilution at which a 1+ score (>90% rounding-up than the control well) was obtained in the majority of the triplicate wells. It was found that concentrations of Tualang honey with 1+ score ranged from 12.5% to 25% (w/v) while concentrations with 2+ to 4+ scores were between 6.25% and 11.25% (w/v). Therefore, the MIC of Tualang honey is 12.5% (w/v). This preliminary study highlighted the potential of honey as an anti-amoebicidal agent.

**Keywords:** Anti-amoebic, *Entamoeba histolytica,* metronidazole, trophozoites.

## Introduction

The use of honey in treating wound and mild bacterial infection has long been known and practiced. Honey added to oral rehydration solution has been found to speed up recovery from vomiting and diarrhea in infants and children suffered from gastroenteritis (Abdul rahman *et al*., 2010). A type of Malaysian honeys, 'Tualang' honey, has been reported to have potential anti-bacterial effect against intestinal bacterial pathogens *in vitro* (Tumin *et al.,* 2005, Tan *et al.,* 2009). Although extensive studies have been carried out on anti-bacterial properties of different types of honey, little was known about their anti-amoebic properties.

Kelantan is one of the endemic states in Peninsular Malaysia for *E. histolytica* infections with faecal oral transmission being the main route of the disease transmission (Zeehaida *et al*., 2009). Metronidazole has long been used for the treatment of amoebiasis, however, inappropriate usage of the drug could lead to drug resistance (Bansal *et al*., 2006; Upcroft and Upcroft, 2001a). Therefore there is a need for safe and effective measures to overcome the problems in the future including the use of local Malaysian honeys as one of the alternative treatments and preventions of amoebiasis in this region. They might share considerably same effectiveness with no side effects, cheap and are easily available as compared to currently available anti-amoebic drugs.

This pilot study was conducted to evaluate the anti-amoebic activity of Tualang honey against *E. histolytica* trophozoites *in vitro*. To our knowledge, this is the first study to reveal the effect of local Malaysian Tualang honey on parasites.

## Materials and Methods

Tualang honey (collected from wild honey bees' hives on *Koompassia excelsa* or tualang tree) was supplied by Federal Agriculture Marketing Authority (FAMA), Malaysia and trophozoites of *E. histolytica* HM1: IMSS (Mexico) strains in TYI-S-33 medium were used in this study.Honey was initially subjected to gamma irradiation at 25 kGy followed by a sterility test before use. Metrogyl Metronidazole (5mg/ml) (JB Chemicals & Pharmaceutical LTD, India) was obtained from Pharmacy Department, Hospital Universiti Sains Malaysia. The anti-amoebic activity of Tualang honey and metronidazole was assessed using broth dilution method with metronidazole as the control or reference. The MIC assays were performed using flat-bottom, sterile 96-wells microtiter tissue culture plates with lid (TPP Techno Plastic Products AG). The procedures were performed in the Biological Safety Cabinet (level II) in the Biomedicine Laboratory, School of Health Sciences, Universiti Sains Malaysia.

### *Procedure for axenic cultivation*

The trophozoites of *E. histolytica* HM1: IMSS strain were axenically cultivated in TYI-S-33 medium in culture flasks and incubated anaerobically. Culture flasks were examined daily using inverted microscope to determine the trophozoite numbers, viability (motility and sticking on the flask surface) and presence of any contamination while the changing of culture media was performed every three days.

### *Preparation of honey and metronidazole for broth dilution*

A 50% (w/v) stock solution of honey was prepared by weighing 10 g of net honey in a known-weight tube (with measuring scale) using the electronic balance and the volume was brought up to 20 ml using TYI-S-33 medium. Honey and medium were mixed well. The freshly prepared stock solution was further diluted with TYI-33 medium to obtain the concentrations of 25, 22.5, 20, 17.5, 15, 12.5, 11.25, 10, 8.75, 7.5 and 6.25% (w/v). Serial dilutions of metronidazole (5mg/mL) using TYI-S-33 medium were also prepared to obtained the concentrations of 100, 50, 25, 12.5, 6.25, 3.2, 1.6, and 0.8 µg/mL.

### *Preparation of MIC assay in well plate*

Trophozoites were harvested from culture flask aseptically before the subsequent cultivation in microtiter well plate was performed. The trophozoites in the flask were chilled on ice for 5 minutes to detach them from the surface of the flask. Medium containing trophozoites was transferred into a 15 mL falcon tube and spun at 2500 rpm for 3 min. The supernatant was discarded and 4 mL of fresh medium were added to the pellet. After mixing, 50 µL of cell suspension were pipetted out into a 1.5 mL eppendorf tube and mixed with an equal volume of 0.4% trypan blue stain for microscopic cell count on Neubauer haemocytometer. Approximately $5x10^4$ cells of *E. histolytica* trophozoites in TYI-S-33 medium were seeded into wells in row A, C, E and G of the 96-well flat-bottom microtiter plate (column 1-11) for Tualang honey MIC assay while the same amount of cells were seeded into wells in row A, C and E (column 3-11) for metronidazole MIC assay. Wells in Row G (for honey MIC assay) and Column 1-2 (for metronidazole MIC assay) were used as blank control wells which contain only TYI-S-33

medium and medium with honey or drug. The volume was brought up to 260 µl (about 80% of well capacity) with fresh TYI-S-33 medium in each well containing trophozoite cells. The plate was incubated overnight at 36 $^0$C under anaerobic condition. When the growth of trophozoites in all wells were confluent (growth covered almost all of well surface), treatments with honey and metronidazole were subsequently performed.

A 130 µL (half of the total 260 µL per well) of medium inside wells containing trophozoites (except control wells which contain medium only) was discarded and replaced with the same volume of different concentration of honey or metronidazole dilutions (ratio of 1:1). Trophozoite growth in each well was monitored microscopically for motility and morphological changes (rounding-up) after 24 hours incubation period by comparing the control and test wells using inverted microscope. All tests were performed in triplicate and were repeated three times or more to obtain reliable results.

Trophozoites growth was scored according to Table 1. MIC was defined as the lowest concentration of honey/metronidazole dilution at which a 1+ score was obtained in the majority of the triplicate wells.

**TABLE 1**: Scoring of trophozoites growth (Upcroft and Upcroft, 2001a)

| Score | Descriptions |
|---|---|
| 1+ | Dead or significantly fewer (not >20% coverage of well surface) and >90% rounded up than the control well. |
| 2+ | 20 – 50% coverage of the well surface and some motility. |
| 3+ | An almost confluent well (>50% coverage of the well surface) and much motility. |
| 4+ | A confluent well (100% coverage of the well surface). |

## Results

For Tualang honey MIC assay, the majority of triplicate wells and repeated assays showed that the minimum concentrations that inhibited the growth of trophozoites (MIC) were 12.5% (w/v) and 6.25 µg/mL for honey and mettonidazole, respectively (**TABLE 2 and 3**).

**TABLE 2**: Scores of MIC assay for Metronidazole (Metrogyl) treatment after 24h incubation

| Column | Concentration (µg/ml) | Assay | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | A | | | B | | | C | | |
| | | *R1 | R2 | R3 | R1 | R2 | R3 | R1 | R2 | R3 |
| 1 | Blank (medium) | | | | | | | | | |
| 2 | Metronidazole blank | | | | | | | | | |
| 3 | Medium with *Eh* | 4+ | 4+ | 3+ | 4+ | 4+ | 3+ | 4+ | 4+ | 4+ |
| 4 | 100 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| 5 | 50 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| 6 | 25 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| 7 | 12.5 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| **8** | **6.25** | **4+** | **4+** | **3+** | **1+** | **1+** | **1+** | **1+** | **1+** | **1+** |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 3.13 | 4+ | 4+ | 4+ | 3+ | 3+ | 3+ | 4+ | 3+ | 3+ |
| 10 | 1.56 | 4+ | 4+ | 3+ | 3+ | 4+ | 4+ | 4+ | 4+ | 3+ |
| 11 | 0.78 | 4+ | 4+ | 3+ | 2+ | 2+ | 3+ | 2+ | 2+ | 2+ |

*R1 = Replicate 1 R2 = Replicate 2 R3 = Replicate 3

**TABLE 3**: Scores of MIC assay for honey treatment after 24h incubation

| Column | % (w/v) | Assay | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | | | B | | | C | | | D | | | E | | |
| | | *R1 | R2 | R3 | R1 | R2 | R3 | R1 | R2 | R3 | R1 | R2 | R3 | R1 | R2 | R3 |
| 1 | 25.00 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| 2 | 22.50 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| 3 | 20.00 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| 4 | 17.50 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| 5 | 15.00 | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ | 1+ |
| **6** | **12.50** | **3+** | **4+** | **3+** | **2+** | **2+** | **2+** | **1+** | **1+** | **1+** | **1+** | **1+** | **1+** | **1+** | **1+** | **1+** |
| 7 | 11.25 | 3+ | 4+ | 4+ | 2+ | 3+ | 2+ | 2+ | 3+ | 3+ | 1+ | 2+ | 2+ | 1+ | 2+ | 2+ |
| 8 | 10.00 | 4+ | 4+ | 4+ | 2+ | 3+ | 2+ | 3+ | 4+ | 3+ | 2+ | 3+ | 2+ | 2+ | 2+ | 2+ |
| 9 | 8.75 | 4+ | 4+ | 4+ | 3+ | 3+ | 2+ | 2+ | 4+ | 3+ | 2+ | 3+ | 3+ | 2+ | 3+ | 3+ |
| 10 | 7.50 | 4+ | 4+ | 3+ | 3+ | 4+ | 2+ | 2+ | 3+ | 2+ | 2+ | 3+ | 3+ | 3+ | 3+ | 3+ |
| 11 | 6.25 | 4+ | 4+ | 3+ | 3+ | 4+ | 2+ | 2+ | 2+ | 2+ | 2+ | 3+ | 4+ | 4+ | 3+ | 3+ |

*R1 = Replicate 1 R2 = Replicate 2 R3 = Replicate 3

## Discussion

Tualang honey is readily available in Malaysia and has been used traditionally for treatment of various diseases partly due to its anti-oxidant and anti-microbial properties. Tualang honey has shown variable but broad spectrum activities against many species of wound and enteric bacteria. *In vitro* anti-bacterial activities of Tualang honey were studied and reported previously (Ainul Hafiza *et al.*, 2005; Tumin *et al*., 2005; Tan *et al*., 2009). However studies on its anti-amoebic properties are still lacking because the culture maintenance and susceptibility test of *E. histolytica* are laborious processes. There are three basic types of culture systems of *E. histolytica*: xenic, monoxenic and axenic. Axenic culture which was performed in the present study is a cultivation of the parasite in the absence of any other metabolizing cells (Clark and Diamond, 2002). Tualang honey used in this research was of research grade. The honey was sterilized earlier using gamma irradiation to get rid of any microbial contaminants especially fungal and clostridial spores and also to prevent the loss of antibacterial activity (Postmes *et al*., 1995; Molan and Allen, 1996).

*In vitro* evaluation of potential anti-bacterial effect of Tualang honey has been carried out by other researchers and similarly we conducted this study to observe its potential in anti-amoebic activities. In the previous study on anti-bacterial activity of Tualang honey, Tan *et al.* (2009) reported that visual MIC values of Tualang honey against 13 wound and enteric bacteria ranged from 8.75% to

25.0% (w/v) while the minimal bactericidal concentration (MBC) values were 20.0% to >25% (w/v). In the present study, Tualang honey was found to have a considerable activity against the chosen strain of *E. histolytica* trophozoites in which the MIC in all replicates ranged from 12.5% to 15.0% (w/v).

MIC assay of the organism against metronidazole ensures reliability of susceptibility test performances. Upon MIC assay of metronidazole against the protozoa, lower MIC values of metronidazole (6.25 µg/mL) was observed when compared to the study by Upcroft and Upcroft (2001b), in which the MIC values of metronidazole against the same strain (HM1:IMSS) of *E. histolytica* trophozoites was reported to be 12.5-25 µg/ml. In another report by Sarker *et al*. (2010), the MIC of metronidazole in their study was <0.8 µg/mL which was sensitive to *E. histolytica* isolates. Adagu *et al*. (2002) have shown the mean 50% inhibitory concentration ($IC_{50}$) value to metronidazole as 18.47 µg/mL for the most susceptible isolates of *E. histolytica* with a >30 µg/mL value as the cut-off value for resistance. The difference in sensitivity pattern of metronidazole against *E. histolytica* could be due to the difference in culture of the parasite, strain of organism, culture medium or raw materials of metronidazole (Sarker *et al*., 2010).

## Conclusion

Tualang honey exhibited an inhibitory effect on *E. histolytica* trophozoites *in vitro*. The results of this pilot study could be a trigger for the evaluation of MIC values of other types of honey in the future. Further study is also needed to explore the potential use of local honeys against *E. histolytica in vivo* as well as studies related to determination of their anti-amoebic properties.

## Acknowledgement

We thanked Dr. Lim Boon Huat School of Health Sciences, USM for providing the stock culture of *E. histolytica* HM1: IMSS strain.

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