# IN VITRO STUDY ON ANTI-AMOEBIC ACTIVITY OF TUALANG HONEY AGAINST *Entamoeba histolytica* TROPHOZOITE

by

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# LIST OF ABBREVIATIONS

$Ca^{2+}$ - Calcium ion

CHO - Chinese hamster ovary

FAC - Ferric Ammonium Citrate

FAMA - Federal Agriculture Marketing Authority

$K_2HPO_4$ - Dipotassium Phosphate

$KH_2PO_4$ - Monopotassium Phosphate or Potassium Dihydrogen Phosphate

MIC - Minimum inhibitory concentration

NaCl - Potassium Chloride

NaOH - Potassium Hydroxide

PPSK - Pusat Pengajian Sains Kesihatan

PPSP - Pusat Pengajian Sains Perubatan

w/v - weight over volume

# ABSTRAK

Sifat anti-bakteria daripada madu Malaysia telah banyak dikaji. Namun, aktiviti anti-ameba daripada madu tempatan terhadap *Entamoeba histolytica* masih belum diketahui. Kajian ini dilakukan untuk menilai aktiviti in vitro madu tualang Malaysia pada pertumbuhan trofozoit *E. histolytica*. Aktiviti anti-ameba madu tualang diuji dengan menggunakan kaedah pencairan dengan *broth* dan metronidazol digunakan sebagai kawalan. Kepekatan madu yang berbeza [6.25% - 25% (berat/isipadu)] dan metronidazol diuji dengan trofozoit strain HM-1: IMSS *E. histolytica* di dalam plat mikro *96-wells* berdasar rata dalam keadaan anaerob. Pertumbuhan trofozoit dalam setiap *well* dinilai menggunakan mikroskop sonsang. Pergerakan dan pembulatan trofozoit dipantau dan nombor skor diberikan. Kepekatan perencatan minimum (MIC) ditakrifkan sebagai kepekatan terendah cairan madu yang mana skor 1+ (>90% *rounded-up trophozoites*) diperoleh pada kebanyakan *well* tiga replikat. Didapati bahawa kepekatan madu tualang dengan skor 1+ adalah antara 12.5% hingga 25% (berat/isipadu), sedangkan kepekatan dengan skor 2+, 3+ dan 4+ adalah antara 6.25% hingga 11.25% (berat/isipadu). Oleh itu, MIC madu tualang adalah 12.5% (berat/isipadu). Kajian ini menunjukkan bahawa madu tualang Malaysia mempunyai kesan perencatan terhadap trofozoit *E. histolytica*. Dengan itu, kajian ini telah menghasilkan maklumat asas sains untuk kajian lebih lanjut mengenai potensi penggunaan madu tempatan terhadap jangkitan *E. histolytica* serta kajian yang berkaitan dengan penentuan sifat anti-ameba yang dimiliki madu tempatan.

# 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 study was conducted to preliminarily evaluate in vitro activity of one local Malaysian honey which is tualang honey on the growth of *E. histolytica* trophozoites. Anti-amoebic activity of tualang honey was assessed using a broth dilution method and metronidazole as the control. Different concentrations of honey [6.25% – 25% (weight/volume)] and metronidazole were tested against HM-1:IMSS strain *E. histolytica* trophozoites in the 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 rounding-up and the numbers were scored accordingly. MIC was defined as the lowest concentration of honey dilution at which a 1+ score (>90% rounded up, than the control well) was obtained in the majority of the triplicate wells. It was found that concentrations of tualang honey with a 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 was 12.5% (w/v). This study showed that the local Malaysian tualang honey has an inhibitory effect on *E. histolytica* trophozoites in vitro. Thus, it can provide a scientific basis for further evaluations on potential use of local honeys against *E. histolytica* infection as well as studies related to determination of their anti-amoebic properties.

# 1.0 INTRODUCTION

Amoebiasis is a disease caused by protozoa known as *Entamoeba histolytica*. The two diseases caused by this organism are in forms of intestinal and extraintestinal amoebiasis. Although many individuals with *E. histolytica* infection are asymptomatic, some of them might develop into extraintestinal disease which has significant morbidity and mortality. Amoebiasis has been one of leading causes of death from parasitic infections especially in developing countries.

The medications recommended to treat confirmed amoebiasis vary with clinical manifestations. Asymptomatic intestinal infection with *E. histolytica* can be treated with luminal amoebicides to eradicate the luminal amoebae, thus preventing subsequent tissue invasion and transmission of cysts but this is not the practice in most developing countries. Tissue amoebicides such as metronidazole are used to treat intestinal and extraintestinal invasive diseases. However treatment with those drugs are not without side effects and furthermore resistance can be one of the problem in treating the patient later.

Traditionally, people used to treat wound with honey due to its medicinal properties. One of them is to prevent microbial growth because of its high sugar content but low water content and acidity. The other factor that determines antimicrobial activity of honey is because of hydrogen peroxide which is produced following dilution resulting from the activation of the enzyme glucose oxidase. Peroxide activity is influenced by the concentration of the compound (White *et al.*, 1963, Bang *et al.*, 2003). Studies in Malaysia have reported antimicrobial properties of local honeys and one of Malaysian honey called 'tualang honey' was found to have potential antibacterial effect against intestinal bacterial pathogens in vitro (Tumin *et al.*, 2005, Tan *et al.*, 2009).

It would be beneficial to change pace toward traditional or natural medicine such as honey in treating infections. Although extensive studies have been carried out on antibacterial properties of different types of honey, to the best of our knowledge little is known about their anti-amoebic properties. Since Kelantan is one of endemic states in Peninsular Malaysia for *E. histolytica* infestations and fecal oral transmission is the main route of disease transmission, therefore it would be of great value if we can use local Malaysian honey as one of the alternative treatments and prevention of transmission of the disease in this region by which they might share considerably same effectiveness but low side effects. Moreover, honey is cheap and easily available as compared to currently available drugs.

Therefore this preliminary study is conducted to evaluate the potential use of one of Malaysian honey (tualang honey) to prevent and treat amoebiasis through the assessment of in vitro anti-amoebic activity of tualang honey against *E. histolytica* trophozoites.

# 2.0 REVIEW OF LITERATURE

## 2.1 *E. histolytica*: Biology and Life Cycle

*E. histolytica* is the only known *Entamoeba* species that causes amoebic colitis and amoebic liver abscess (ALA).

On the parasite cultures the trophic amoeba multiply by simple binary fission as they do in their natural host. The cyst of amoeba consists of 4 nuclei (quadrinucleate cyst). Through a perforation in the wall, the amoeba escapes from the cyst and is called 4-nucleate excysted or metacystic amoeba which later produce new generation of trophic form by complicated series of nuclear and cytoplasmic divisions. Final product of further division are eight uninucleate amoebulae by each quadrinucleate amoeba hatched from a cyst. Amoebulae are young trophozoites and not gametes or conjugants (Dobell, 1928).

*E. histolytica* exists in two forms; the dividing form, trophozoite and the dormant form, the cyst. Infection to human begins with the ingestion of the cyst which present in food or water contaminated with human fecal material. In the stomach, the cysts survive the acidic pH environment and pass into the intestine. Cysts undergo excystment in the ileo-cecal region and each cyst gives rise to eight trophozoites. These trophozoites then migrate to and multiply in the colon. Usually, trophozoites in the intestine live as commensals. However, it is very rare for trophozoites to attack and invade the intestinal mucosa causing dysentery or progress through the blood vessels to extra-intestinal locations like liver, brain and lungs, where they may form abscesses which can be fatal. Many of the trophozoites encyst in the intestine and produce quadrinucleated cysts. Both trophozoites and cysts are excreted along with the feces. Cysts can survive for prolonged periods outside the host while the trophozoites survive only a short period of time. Trophozoites do not involve in transmission of the disease but are responsible for pathogenesis of the disease. The reservoir of human infection is

termed as "carrier" or asymptomatic human host who continuously passes cysts (Sehgal *et al.*, 1996).

## 2.2 Pathogenesis of Amoebic Infection

The major limitation in studying pathogenesis is the lack of a satisfactory animal model which can duplicate the spectrum of human disease. However, several species have been used as animal models to study various aspects of pathogenesis (Meerovitch and Chadee, 1988). Hamsters and gerbils are most commonly used as models for liver disease. When trophozoites are injected directly into the liver of these animals, lesions are produced. In vitro models are also available for studying various steps involved in pathogenesis (Petri and Ravdin, 1988).

Based on a study conducted by Sehgal *et al.*, (1996), there are several stages involve in the pathogenesis of infection by *E. histolytica* which are listed below;

### (A) Colonization and interaction with the intestinal flora

Trophozoites interact with the intestinal flora in the guts. It was stated that trophozoites undergo changes when interact with bacteria (Sehgal *et al.*, 1996). Non-virulent axenic *E. histolytica* can regain its virulence when associated with bacteria such as *Escherichia coli, Salmonella typhosa or S. paratyphi.*

### (B) Adherence to establish direct contact between trophozoite and target cell

Trophozoites need to adhere to target cells for cytotoxicity effect. Observation on cinemicrography has shown that the CHO cells in direct contact with amoeba displayed membrane blebbing and release from cover slip. Those not in direct contact with amoeba however, stayed viable. A mixture of CHO cells and trophozoites in presence of high molecular weight dextran (10%) did not trigger lysis because trophozoites were prevented from adhering due to the dextran (Ravdin and Guerrant, 1981).

**(C) Lysis of target cells by release of toxins**

Invasion of *E. histolytica* to the mucosal layer leads to depletion of mucous and disruption of epithelial barrier. Lysis of target cell requires amoebic microfilament function, $Ca^{2+}$ flux and phospholipase A. Thiol-proteases are believed to be involved in pathogenesis of disease. Trophozoites may gain acces to target cells aided by the proteases which degrade the extracellular matrix. Pore-forming proteins may be responsible for cytolysis. Dodson and Petri, (1994) in their study stated that purified amoebic pore-forming proteins lysed erythrocytes and created pores in lipid bilayers.

**(D) Phagocytosis**

Trophozoites with ingested erythrocytes are higher in stools of invasive amoebiasis patients than those of healthy human carrier. Mammalian tissue culture cells which were grown and undergo phagocytosis by amoeba when observed with transmission electron microscope. Amoeba phagocytes only cells with intact plasma membrane showing that prior cell lysis is not required for endocytosis (McCaul, 1977). A phagocytosis-deficient mutant of *E. histolytica* which was isolated by Orozco *et al.*, (1983) was found to be poor in phagocytosis and low in virulence, when tested in the hamster liver model. Thus, there seems to be a correlation between phagocytosis and virulence.

## 2.3 Prevalence of Amoebic Infection

Epidemiologically, amoebic infection predominantly affects individuals of lower socioeconomic status who live in developing countries. Prevalence of amoebic infection depends on several factors such as cultural habits, level of sanitation, crowding, socioeconomic status and age (Ravdin, 1995). Study shows that prevalence is increased among school-aged children. This statement is supported by prevalence studies of protozoan parasites carried mainly amongst school children in Peninsular Malaysia (Noorhayati *et al.*, 1981, Hamimah *et al.*, 1982, Sinniah and Rajeswari, 1988).

**Table 2.1: Prevalence of Amoebiasis in Malaysia**

| Author & Year | Prevalence (%) of Infection |
|---|---|
| Bisseru & Aziz (1970) | 1.5 |
| Dunn (1972) | 5.1 |
| Dissanaike *et al* (1977) | 8.7 |
| Sinniah *et al* (1978) | 1.3 |
| Nawalinski & Roundy (1978) | 1.2 |
| Hamimah *et al* (1982) | 2.3 |
| Thomas & Sinniah (1982) | 8.3 |
| Sinniah (1984) | 4.4 |
| Che Ghani *et al* (1987) | 14.4 |
| Noor Hayati *et al* (1998) | 11.5 |

* Adapted from Norhayati *et al.*, (2003).

In 1986, a study on immunodiagnosis and seroepidemiology of amoebiasis carried out in Selangor, Malaysia stated that areas in Selangor were endemic for amoebiasis and the prevalence was high in older groups (Thomas and Leng, 1986). Another study of amoebic serodiagnosis using Indirect Haemagglutination Assay (IHA) to detect anti-*Entamoeba histolytica* antibodies in patient's serum sample concluded that Kelantan is also considered as one of endemic areas for amoebiasis (Zeehaida *et al.*, 2008).

Study of frequency of *Entamoeba histolytica* and *Entamoeba dispar* infection was held in a rural community in the state of Morelos, Mexico, through PCR technique using specific primer. The *E. histolytica* was detected in 33 of 290 analyzed stool samples (11.4%), *E. dispar* was observed in 21 samples (7.2%) and both species of *Entamoeba* were detected in seven samples (2.4%). So a higher *E. histolytica* than *E. dispar* infection frequency was detected (13.8 versus 9.6%) (Ramos *et al.*, 2005).

**2.4 Clinical Manifestation**

Intestinal amoebiasis could present as asymptomatic cyst passage, acute amoebic rectocolitis, chronic non-dysenteric colitis, and amoeboma. Clinical history of abdominal pain, tenesmus, and frequent loose, watery stools containing blood and mucus were usually presented in acute amoebic colitis patients. Only few patients were febrile and had diarrhea that cause clinically significant dehydration. Extraintestinal amoebiasis such as amoebic liver abscess (ALA) were associated with leukocytosis without eosinophilia, mild anemia, elevated levels of alkaline phosphatase, elevated levels of transaminase in cases of severe disease, and a high erythrocyte sedimentation rate (Ravdin, 1995).

**2.5 Diagnosis**

Diagnosis of intestinal amoebiasis relies commonly on microscopic examination of stool samples for the presence or absence of *E. histolytica* (Tanyuksel and Petri, 2003, Ravdin, 1995).

Evaluation of patients sonographically in diagnosis of amoebic liver abscess (ALA) has revealed that 16% cases had multiple abscesses, 35% had an abscess in the left lobe, and the remaining 49% had a solitary abscess in the right lobe (Sharma *et al.*, 2003). Later in their recent study found that 22% cases had multiple abscesses, 13% cases had a solitary abscess in the left lobe, and 65% of cases had abscess in

single right lobe. They also found that the gold standard for diagnosing liver abscesses was abdominal ultrasound (Sharma *et al.*, 2010).

Amoebiasis complication such as acute fulminant necrotizing amoebic colitis is very rare and hard to diagnose and treat. It is sometimes confused with idiopathic inflammatory bowel disease which resulted in wrong steroids administration. Diagnostic methods such as colonoscopic appearance and colonic tissue biopsy are helpful in differentiating amoebiasis from other forms of colitis (Gupta *et al.*, 2009).

**2.6 Treatment**

Metronidazole has been used as one of the drugs to treat intestinal and extraintestinal invasive diseases for years (Ravdin, 1995, Wassmann *et al.*, 1999, Pal *et al.*, 2009). Although it becomes the choice of treatment for amoebic infection, resistance to metronidazole would occur (Wassmann *et al.*, 1999, Sarker *et al.*, 2010).

Samarawickrema *et al.*, (1997) was first to described the in vitro induction of metronidazole resistance under microaerophilic conditions in the protozoan parasite *E. histolytica*.

## 2.7 Previous Studies on Anti-amoebic Activities

Anti-amoebic activity of methanol extract of mature seeds of *Carica papaya* was tested in vitro on axenic culture of *E. histolytica* using metronidazole as a reference amoebicidal agent. The MIC of seed extract was > 62.5 µg/mL as compared to < 0.8 µg/mL for metronidazole. The study suggested that the mature seeds of *Carica papaya* had anti-amoebic effect but was less pronounced than metronidazole (Sarker *et al.*, 2010).

More than a decade ago, the anti-amoebic effect of a crude drug formulation of herbal extracts against *E. histolytica* in vitro and in vivo was evaluated. In the traditional system of medicine in India, the formulation has been prescribed for intestinal disorders. It comprises of five medicinal herbs, namely, *Boerhavia diffusa, Berberis aristata, Tinospora cordifolia, Terminalia chebula* and *Zingiber officinale*. The dried and pulverized plants were extracted in ethanol together and individually. In vitro amoebicidal activity was studied to determine the minimal inhibitory concentration (MIC) values of all the constituent extracts as well as the whole formulation. The formulation had a MIC of 1000 µg/ml as compared with 10 µg/ml for metronidazole. In experimental caecal amoebiasis among rats the formulation had a curative rate of 89% with the average degree of infection (ADI) reduced to 0.4 in a group dosed with 500 mg/kg per day as compared with ADI of 3.8 for the sham-treated control group of rats. Metronidazole had a cure rate of 89% (ADI = 0.4) at a dose of 100 mg/kg per day and cured the infection completely (ADI = 0) when the dosage was doubled to 200 mg/kg per day (Sohni *et al.*, 1995).

Several in vitro and in vivo features of both virulent *E. histolytica* (v*Eh*) and non-virulent *E. histolytica* (nv*Eh*) axenic HM-1:IMSS strains were compared, such as complement resistance, proteinase activity, haemolytic, phagocytic and cytotoxic capacities, survival in mice caecum, and susceptibility to $O_2$. The only difference

observed was a higher in vitro susceptibility of nv*Eh* to $O_2$. The molecular mechanism of that difference was analyzed in both groups of amoebae after high $O_2$ exposure. v*Eh* $O_2$ resistance correlated with: (i) higher $O_2$ reduction ($O_2$ and $H_2O_2$ production); (ii) increased $H_2O_2$ resistance and thiol peroxidase activity, and (iii) reversible pyruvate: ferredoxin oxidoreductase (PFOR) inhibition. Despite the high level of carbonylated proteins in nv*Eh* after $O_2$ exposure, membrane oxidation by reactive oxygen species was not observed. These results suggest that the virulent phenotype of *E. histolytica* is related to the greater ability to reduce $O_2$ and $H_2O_2$ as well as PFOR reactivation, whereas nv*Eh* undergoes irreversible PFOR inhibition resulting in metabolic failure and amoebic death (Ramos-Martínez *et al.*, 2009).

Wassmann *et al.*, (1999) discovered that cultivation of wild-type *E. histolytica* trophozoites (HM-1) under microaerophilic conditions (5–7% oxygen) in the presence of various amounts of metronidazole revealed that a concentration of 12 μM was sufficient to reproducibly kill 50% of the cells within 24 hours and was lethal for all cells after prolonged exposures over 72 hours. Meanwhile, amoebae grown under strict anaerobic conditions were less susceptible to metronidazole. As for clinical point of view, only those *E. histolytica* trophozoites that are resistant to metronidazole in the presence of oxygen are of medical importance.

**2.8 An Overview of Malaysian Tualang Honey**

Malaysian tualang honey got its name from the tualang tree (*Koompassia excelsa*) where the beehives resided. The honey is collected from the combs of Asian rock bees (*Apis dorsata*). Tualang honey is used commonly as food in Malaysia. But, there are few scientific information in publication until now (Tan *et al.*, 2009).

Recently, local researchers had successfully detected and identified a total of 35 volatile compounds in tualang honey by using gas chromatography method. About half of tualang honey composition was hydrocarbon compounds. The highest

compound was methanol and compounds such as acetic, palmitic and stearic acids as well as 2-furancarboxaldehyde, furfural alcohol and 5-(Hydroxymethyl) furfural (HMF). Other compounds that had not been reported previously were also detected in tualang honey (Syazana *et al.*, 2010).

Former study by White *et al.* (1963), included an experiment on production of hydrogen peroxide from honey. It proves that inhibine which was identified as antibacterial material was hydrogen peroxide produced by glucose oxidase enzyme. The presence of presumed inhibine, hydrogen peroxide did inhibit the growth of tested microorganism, *Staphylococcus aureus* in the inhibine assay. They also concluded that molds and yeast were not sensitive to hydrogen peroxide compared to bacteria.

Study by Postmes *et al.* (1995) show that antibacterial activity of honey remained unaltered after an irradiation dose of 25 kGy and assumed that glucose oxidase enzyme which involved in hydrogen peroxide production cannot be damaged by irradiation easily. They also discovered that amylase activity in European honey was reduced after irradiation in their study. But it has not reported in Chinese or Australian honey and the reason has yet to be known.

# 3.0 OBJECTIVES

General objective:

- The objective of the study is to evaluate the in-vitro anti-amoebic activity of tualang honey on *E. histolytica* trophozoites.

Specific objectives:

- To observe the anti-amoebic effect of tualang honey on the growth of trophozoites of HM-1:IMSS strain of *E. histolytica* in the microtitre plate.
- To describe the minimum inhibitory concentration (MIC) range of tualang honey against axenic culture of *E. histolytica* trophozoite using metronidazole as a reference amoebicidal agent.

# 4.0 METHODOLOGY

## 4.1 Study Design

This is a pilot study involving selected strain of *E. histolytica* and assessment of anti-amoebic activity of tualang honey using a broth dilution method with metronidazole as the control or reference.

## 4.2 Equipment and Instrument

The equipments and instruments that have been used during research project are listed in the table below and can be found in Biomedicine Laboratory (PPSK) and Microbiology Laboratory (PPSP).

**Table 4.1: Equipment & instrumentation used**

| No. | Item |
|---|---|
| 1 | Incubator |
| 2 | Icemaker machine |
| 3 | Refrigerator (4$^0$C) |
| 4 | Freezer (-20$^0$C) |
| 5 | Biological Safety Cabinet (BSC) Level II |
| 6 | Water bath |
| 7 | Autoclave machine |
| 8 | Centrifuge machine |
| 9 | Anaerobic jar system (Oxoid) |
| 10 | Micropipette |
| 11 | Schott bottle |
| 12 | Beaker |
| 13 | pH balance machine |
| 14 | Magnetic stirrer |
| 15 | Electronic balance |
| 16 | Ultrabalance |
| 17 | Inverted microscope |
| 18 | Neubauer haemocytometer |
| 19 | Counter |
| 20 | Bunsen burner |

## 4.3 Materials

**Table 4.2: Materials used**

| No. | Material |
|---|---|
| 1 | Metrogyl Metronidazole (5 mg/ml) (JB Chemicals & Pharmaceutical LTD, India) |
| 2 | Trypan Blue Stain 0.4% solution |
| 3 | Sterile 96-wells microtitre tissue culture plate with lid (TPP Techno Plastic Products AG) |
| 4 | Eppendorf tube |
| 5 | Centrifuge tube |
| 6 | Parafilm seal |
| 7 | Tips (blue & yellow) |
| 8 | Gloves |
| 9 | Distilled water |
| 10 | Biosate peptone (BBL™ Becton, Dickinson and Company) |
| 11 | Dextrose |
| 12 | $K_2HPO_4$ |
| 13 | $KH_2PO_4$ |
| 14 | NaCl |
| 15 | Cysteine |
| 16 | Ascorbic Acid |
| 17 | FAC |
| 18 | NaOH |
| 19 | Diamond Vitamin Tween 80 solution (SAFC Biosciences) |
| 20 | Bovine serum (GIBCO™ Invitrogen Corporation) |
| 21 | Filter paper |

## 4.4 Medium Preparation

Medium used in this research for axenic culture of *E. histolytica*, broth dilutions, and MIC assay was TYI-S-33 medium. The procedure for preparation of 1 litre TYI-S-33 medium is listed below:

### A. Solution A preparation

Biosate peptone 30 g and dextrose 10 g were weighed using the electronic balance and dissolved in 500 ml of distilled water and labelled as solution A.

**B. Solution B preparation**

370 ml of solution B was prepared by weighing materials listed below using ultrabalance and dissolved in distilled water.

**Table 4.3: Materials for solution B**

| Material | Weight (g) |
|---|---|
| $K_2HPO_4$ | 1.0 |
| $KH_2PO_4$ | 0.6 |
| NaCl | 2.0 |
| Cysteine | 1.0 |
| Ascorbic acid | 0.2 |

1. Solution A and B were mixed and 22.5 mg FAC was added.

2. The pH of medium solution was adjusted to 6.8 with 2M NaOH.

3. The medium solution was filtered and autoclaved (121$^0$C for 10 minutes).

4. The autoclaved medium was left cooling in drawer for overnight.

5. 30 ml of vitamin solution and 100 ml of bovine serum were added and mixed well and ready to be used.

6. Medium was stored in -20$^0$C freezer if not used for long period of time and kept in 4$^0$C refrigerator for daily usage.

**4.5 Maintenance of Parasite Cell Culture**

The trophozoites of *E. histolytica* strain HM-1:IMSS were cultivated in Biomedicine Laboratory, School of Health Sciences. The trophozoites were axenically cultured in TYI-S-33 medium. The trophozoites culture was maintained for every three days by changing the culture medium with new medium. During maintenance of culture, culture tubes were examined for viability (indicate the trophozoites were alive)

by observation using inverted microscope. Trophozoites stock culture with too much growth of cells would be expanded by sub-culturing each of the culture tubes and became the new stock culture. Whenever trophozoites were used for research purpose and culture tubes were depleted, then sub-culture of stock culture was made to increase the stock culture and also ready for upcoming research usage. Maintenance procedure was conducted under sterile condition (BSC level II setting) and aseptic technique (Bunsen burner).

### 4.6 Preparation of Honey Broth Dilutions

Broth dilution method was used to prepare honey stock solution and the procedure for the preparation was adapted from Tan *et al.*, (2009) with some modification being made to suit this research. Tualang honey was obtained from Pharmacology Department, School of Medical Sciences. This Tualang honey was supplied by FAMA and given to USM for research purposes.

#### 4.6.1 Fifty percent (weight/volume) stock solution

Fifty percent (weight/volume) stock solution of honey was prepared by weighing 10 g of net honey in known-weight tube (with scale presence for measurement) using the electronic balance and the volume was brought up to 20 ml using TYI-S-33 medium. The mixture of honey and medium was shaken (not vigorously) until they mixed well.

#### 4.6.2 Further dilutions

Simple calculations were carried out in order to prepare further dilutions from the 50% (w/v) stock solution. The fresh prepared 50% (w/v) stock solution was further diluted with TYI-S-33 medium to obtain honey dilutions with concentration of 25, 22.5, 20, 17.5, and 15% (w/v). After that, from the five dilutions earlier, further serial dilutions were made and obtained new honey dilutions with concentration of 12.5, 11.25, 10,

8.75, 7.5, and 6.25% (w/v) were obtained. Then, all newly made honey dilutions were aliquot into 1.5 ml eppendorf tubes ready for use in MIC assay. Stock solution and dilutions of honey were stored in $4^0$C refrigerator or cold room for preservation. Net honey (undiluted) was stored in room temperature. All procedures involved in preparation of honey broth dilutions were conducted under sterile condition and using aseptic technique.

### 4.7 Preparation of MIC Assay Well-Plate

#### 4.7.1 Harvesting the trophozoites and cell suspension preparation

Trophozoites need to be harvested from culture tubes before being cultured in microtiter well-plate. Medium containing trophozoites in culture tubes was poured into centrifuge tube and chilled in ice bath for about five minutes. Then, the trophozoite-containing tube was centrifuged for three minutes at 2500 RPM. After centrifugation, the tube was again chilled in ice bath. The supernatant was discarded and 4 ml of fresh medium was pipetted into the tube containing the trophozoites pellet. By using the tip of micropipette, the pellet and medium were mixed until the cell suspension was ready to be used. After that, 50 µl of cell suspension was pipetted out into 1.5 ml eppendorf tube and proceeded to cell count. These techniques were handled aseptically.

#### 4.7.2 Trophozoite cell count and cell volume determination

1. Cover-slip and haemocytometer were cleaned, if necessary using alcohol.

2. A cover-slip was then fixed onto the haemocytometer.

3. Equal volume of 0.4% trypan blue stain was mixed well together (not too vigorous) with 50 µl cell suspension inside eppendorf tube earlier.

4. Haemocytometer grid was visualised under inverted microscope and position of large corner square was determined.

5. At the large corner square, viable trophozoites (live) were counted with counter. Counting was continued until all four large corner squares were counted for live cells. Dead cells were excluded from counting because only live cells were used in this research (Dead cells were stained blue while live cells were colourless and bright).

6. Concentration of cells was determined by using formula below:

No. of cells counted X 5000 = No. of cells in 1 ml cell suspension (cells/ml)

Thus, total cells in cell suspension = No. of cells in 1 ml X Volume of cell suspension (ml).

7. Volume of $5x10^4$ trophozoite cells was determined by using formula below:

No. of cells in 1 ml (cells/ml) = $5x10^4$ cells / volume of $5x10^4$ cells (ml)

Thus, volume of $5x10^4$ cells (ml) = $5x10^4$ cells / No. of cells in 1 ml (cells/ml)

**4.7.3 Seeding trophozoites into culture plate**

A sample of $5x10^4$ trophozoite cells were seeded into the culture plate in 4 rows. First row until third row (Row A, C, and E) were the triplicate wells where different concentration of honey dilutions to be tested while the fourth row (Row G) reserved for control wells. For triplicate wells, trophozoite cells were seeded into column 1 – 11 while column 3 and 4 for the control wells. Then, the volume was brought up to 260 µl (about 80% of well capacity) with fresh TYI-S-33 medium for each well containing trophozoite cells. Meanwhile for control wells, column 1 and 2 which have no cells inside were filled with medium and later became blank control (medium only and medium with honey respectively). The culture plate was closed with lid and sealed with parafilm and tape (if necessary). Culture plate then observed under inverted microscope to ensure trophozoite cells were seeded well. Lastly, culture plate was incubated inside standard incubator at 36±0.5$^0$C for overnight. Procedure of seeding trophozoites was done under sterile condition and using aseptic technique.

### 4.7.4 MIC determination of metronidazole drug

Meanwhile, MIC of metronidazole drug was first determined as the drug would be used as reference for inhibited growth of trophozoites culture inside control wells. Firstly, stock metronidazole (5 mg/ml) was diluted several times with TYI-S-33 medium to obtained concentration 200 µg/ml. Then, serial dilutions were done to obtained several dilutions: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 µg/ml. For MIC assay of metronidazole, only 1$^{st}$ – 3$^{rd}$ rows were used and no 4$^{th}$ row was being used as for control wells in MIC assay of honey. Both column 1 and 2 were used as blank wells (medium only and medium with metronidazole respectively). Column 3 – 11 were seeded with trophozoites and treated later with metronidazole of concentration 100 – 0.78 µg/ml respectively starting from column 4. Ratio of trophozoite medium to drug is 1:1 (130 µl trophozoite medium and 130 µl drug). Growth of trophozoites was evaluated after 24 hours incubation and MIC was also determined.

### 4.7.5 Culture treatment with honey dilutions

After overnight incubation, culture plate was monitored for trophozoites growth inside wells using an inverted microscope. When all of the trophozoites-containing wells were well grown and confluent (growth covered almost all of well surface), treatment with honey proceeded. Culture treatment was also handled aseptically under sterile condition. By using micropipette, 130 µl (half of 260 µl) of medium inside wells containing trophozoites (except control wells) was discarded and replaced with same volume of different concentrations of honey dilutions (Ratio of 1:1). 130 µl of honey dilutions: 25, 22.5, 20. 17.5, 15, 12.5, 11.25, 10, 8.75, 7.5, and 6.25% (w/v) were filled into 1$^{st}$ – 3$^{rd}$ rows (column 1 – 11 respectively). For control wells, the 4$^{th}$ row, column 1 was left blank (medium only), column 2 replaced for honey blank (medium with honey only), column 3 contain medium with trophozoites only (growth well), and column 4 contain medium with trophozoites and metronidazole drug (inhibited growth).

Finally, the culture plate was covered with lid, sealed with parafilm and incubated again for 24 hours at 36±0.5$^0$C. The assay was then evaluated microscopically and aided by adapted trophozoites scoring system with a few modifications.

### 4.8 Evaluation of MIC Assay

The evaluation procedure for MIC assay was adapted from Upcroft and Upcroft, (2001). The trophozoites culture grown in 96-wells microtiter plate was microscopically observed after 24 hours incubation period by using inverted microscope and was compared to control wells for scoring to be done. Trophozoites growth was scored using the table shown below:

**Table 4.4: Scoring of trophozoites growth**

| 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). |

Trophozoites were monitored for motility and rounding-up, which was an indication of susceptibility to honey. Rounding-up referred to the ball-like *E. histolytica* trophozoite with no evidence of purposeful movement and no pseudopodia. All scores were recorded into observation data table. MIC was defined as the lowest concentration of honey at which a 1+ score was obtained in the majority of the triplicate wells. MIC assay was repeated five times for reliable results.

# 5.0 RESULTS

## 5.1 MIC assay: Metronidazole (Metrogyl)

**Table 5.1: Scores for MIC assay of Metronidazole (Metrogyl) after 24h incubation.**

| Assay | Column | Concentration (µg/ml) | Replicate | | | MIC |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | |
| A | 1 | Blank (medium) | | | | 12.5 µg/ml |
| | 2 | Metronidazole blank | | | | |
| | 3 | Medium with *Eh* | 4+ | 4+ | 3+ | |
| | 4 | 100 | 1+ | 1+ | 1+ | |
| | 5 | 50 | 1+ | 1+ | 1+ | |
| | 6 | 25 | 1+ | 1+ | 1+ | |
| | 7 | 12.5 | 1+ | 1+ | 1+ | |
| | 8 | 6.25 | 4+ | 4+ | 3+ | |
| | 9 | 3.13 | 4+ | 4+ | 4+ | |
| | 10 | 1.56 | 4+ | 4+ | 3+ | |
| | 11 | 0.78 | 4+ | 4+ | 3+ | |
| B | 1 | Blank (medium) | | | | 6.25 µg/ml |
| | 2 | Metronidazole blank | | | | |
| | 3 | Medium with *Eh* | 4+ | 4+ | 3+ | |
| | 4 | 100 | 1+ | 1+ | 1+ | |
| | 5 | 50 | 1+ | 1+ | 1+ | |
| | 6 | 25 | 1+ | 1+ | 1+ | |
| | 7 | 12.5 | 1+ | 1+ | 1+ | |
| | 8 | 6.25 | 1+ | 1+ | 1+ | |
| | 9 | 3.13 | 3+ | 3+ | 3+ | |
| | 10 | 1.56 | 3+ | 4+ | 4+ | |
| | 11 | 0.78 | 2+ | 2+ | 3+ | |
| C | 1 | Blank (medium) | | | | 6.25 µg/ml |
| | 2 | Metronidazole blank | | | | |
| | 3 | Medium with *Eh* | 4+ | 4+ | 4+ | |
| | 4 | 100 | 1+ | 1+ | 1+ | |
| | 5 | 50 | 1+ | 1+ | 1+ | |
| | 6 | 25 | 1+ | 1+ | 1+ | |
| | 7 | 12.5 | 1+ | 1+ | 1+ | |
| | 8 | 6.25 | 1+ | 1+ | 1+ | |
| | 9 | 3.13 | 4+ | 3+ | 3+ | |
| | 10 | 1.56 | 4+ | 4+ | 3+ | |
| | 11 | 0.78 | 2+ | 2+ | 2+ | |

Majority of triplicate wells and repeated assays shows that the minimum concentration of metronidazole which inhibited the growth of trophozoites (MIC) was 6.25 µg/ml.

## 5.2 MIC Assay: Honey Treatment

**Table 5.2: Scores for MIC assay of honey treatment after 24h incubation.**

| Assay | Column | Concentration [% (w/v)] | Replicate | | | MIC |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | |
| A | 1 | 25.00 | 1+ | 1+ | 1+ | 15 % (w/v) |
| | 2 | 22.50 | 1+ | 1+ | 1+ | |
| | 3 | 20.00 | 1+ | 1+ | 1+ | |
| | 4 | 17.50 | 1+ | 1+ | 1+ | |
| | 5 | 15.00 | 1+ | 1+ | 1+ | |
| | 6 | 12.50 | 3+ | 4+ | 3+ | |
| | 7 | 11.25 | 3+ | 4+ | 4+ | |
| | 8 | 10.00 | 4+ | 4+ | 4+ | |
| | 9 | 8.75 | 4+ | 4+ | 4+ | |
| | 10 | 7.50 | 4+ | 4+ | 3+ | |
| | 11 | 6.25 | 4+ | 4+ | 3+ | |
| Control | 1 | Blank (TYI-S-33 ONLY) | | - | | |
| | 2 | Blank (TYI-S-33 + Honey) | | - | | |
| | 3 | *Eh* ($5X10^4$ trophozoites) | | 3+ | | |
| | 4 | *Eh* + Metronidazole (25µg/mL) | | 1+ | | |
| B | 1 | 25.00 | 1+ | 1+ | 1+ | 15 % (w/v) |
| | 2 | 22.50 | 1+ | 1+ | 1+ | |
| | 3 | 20.00 | 1+ | 1+ | 1+ | |
| | 4 | 17.50 | 1+ | 1+ | 1+ | |
| | 5 | 15.00 | 1+ | 1+ | 1+ | |
| | 6 | 12.50 | 2+ | 2+ | 2+ | |
| | 7 | 11.25 | 2+ | 3+ | 2+ | |
| | 8 | 10.00 | 2+ | 3+ | 2+ | |
| | 9 | 8.75 | 3+ | 3+ | 2+ | |
| | 10 | 7.50 | 3+ | 4+ | 2+ | |
| | 11 | 6.25 | 3+ | 4+ | 2+ | |
| Control | 1 | Blank (TYI-S-33 ONLY) | | - | | |
| | 2 | Blank (TYI-S-33 + Honey) | | - | | |
| | 3 | *Eh* ($5X10^4$ trophozoites) | | 4+ | | |
| | 4 | *Eh* + Metronidazole (25µg/mL) | | 1+ | | |

**Table 5.2, continued**

| | | | | | | |
|---|---|---|---|---|---|---|
| C | 1 | 25.00 | 1+ | 1+ | 1+ | 12.5 % (w/v) |
| | 2 | 22.50 | 1+ | 1+ | 1+ | |
| | 3 | 20.00 | 1+ | 1+ | 1+ | |
| | 4 | 17.50 | 1+ | 1+ | 1+ | |
| | 5 | 15.00 | 1+ | 1+ | 1+ | |
| | 6 | 12.50 | 1+ | 1+ | 1+ | |
| | 7 | 11.25 | 2+ | 3+ | 3+ | |
| | 8 | 10.00 | 3+ | 4+ | 3+ | |
| | 9 | 8.75 | 2+ | 4+ | 3+ | |
| | 10 | 7.50 | 2+ | 3+ | 2+ | |
| | 11 | 6.25 | 2+ | 2+ | 2+ | |
| Control | 1 | Blank (TYI-S-33 ONLY) | | - | | |
| | 2 | Blank (TYI-S-33 + Honey) | | - | | |
| | 3 | *Eh* ($5X10^4$ trophozoites) | | 2+ | | |
| | 4 | *Eh* + Metronidazole (25µg/mL) | | 1+ | | |
| D | 1 | 25.00 | 1+ | 1+ | 1+ | 12.5 % (w/v) |
| | 2 | 22.50 | 1+ | 1+ | 1+ | |
| | 3 | 20.00 | 1+ | 1+ | 1+ | |
| | 4 | 17.50 | 1+ | 1+ | 1+ | |
| | 5 | 15.00 | 1+ | 1+ | 1+ | |
| | 6 | 12.50 | 1+ | 1+ | 1+ | |
| | 7 | 11.25 | 1+ | 2+ | 2+ | |
| | 8 | 10.00 | 2+ | 3+ | 2+ | |
| | 9 | 8.75 | 2+ | 3+ | 3+ | |
| | 10 | 7.50 | 2+ | 3+ | 3+ | |
| | 11 | 6.25 | 2+ | 3+ | 4+ | |
| Control | 1 | Blank (TYI-S-33 ONLY) | | - | | |
| | 2 | Blank (TYI-S-33 + Honey) | | - | | |
| | 3 | *Eh* ($5X10^4$ trophozoites) | | 4+ | | |
| | 4 | *Eh* + Metronidazole (25µg/mL) | | 1+ | | |

**Table 5.2, continued**

| | | | | | | |
|---|---|---|---|---|---|---|
| E | 1 | 25.00 | 1+ | 1+ | 1+ | 12.5 % (w/v) |
| | 2 | 22.50 | 1+ | 1+ | 1+ | |
| | 3 | 20.00 | 1+ | 1+ | 1+ | |
| | 4 | 17.50 | 1+ | 1+ | 1+ | |
| | 5 | 15.00 | 1+ | 1+ | 1+ | |
| | 6 | 12.50 | 1+ | 1+ | 1+ | |
| | 7 | 11.25 | 1+ | 2+ | 2+ | |
| | 8 | 10.00 | 2+ | 2+ | 2+ | |
| | 9 | 8.75 | 2+ | 3+ | 3+ | |
| | 10 | 7.50 | 3+ | 3+ | 3+ | |
| | 11 | 6.25 | 4+ | 3+ | 3+ | |
| Control | 1 | Blank (TYI-S-33 ONLY) | | - | | |
| | 2 | Blank (TYI-S-33 + Honey) | | - | | |
| | 3 | *Eh* ($5X10^4$trophozoites) | | 4+ | | |
| | 4 | *Eh* + Metronidazole (25µg/mL) | | 1+ | | |

Majority of triplicate wells and repeated assays shows that the minimum concentration of honey dilution which inhibited the growth of trophozoites (MIC) was 12.5% (w/v).

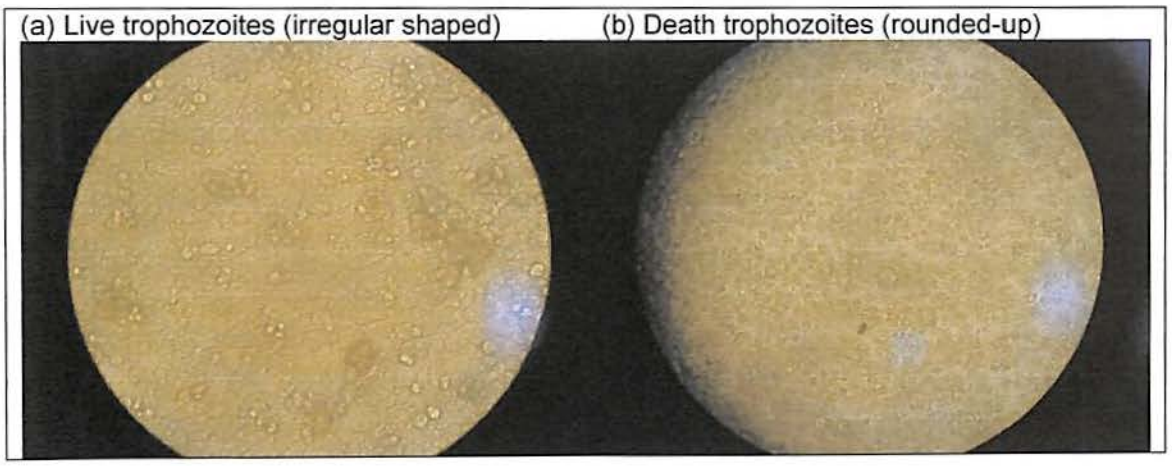


Plate 5.1: Microscopic visualization of *E. histolytica* trophozoites under inverted microscope. 200X magnification power.