Faculty of Resource Science and Technology

DEVELOPMENT OF TEST METOHD FOR ANTI-AMOEBIC ACTIVITY

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Development of Test Method for Anti-amoebic Activity

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List of Abbreviations

CC	Cytotoxicity control
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
FLA	Free-living Amoebae
LSD	Fisher Least Significant Diffenrence test
NA	Nutrient agar
NB	Nutrient broth
NNA	Non-nutrient agar
PAS	Page’s modified Neff’s amoeba saline
PBS	Phosphate buffered solution
PCR	Polymerase Chain Reaction
RBC	Red blood cells
TC	Thiourea control
TD	Amino acid-thiourea derivative
WHO	World Health Organization

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ABSTRACT

This study was carried out to obtain preliminary data of anti-amoebic screening test using non-pathogenic free living amoebae (FLA) and thus develop an appropriate preliminary test method for anti-amoebic activity. The anti-amoebic screening test was performed by treating free living amoebae with amino acid-thiourea derivative at concentration of 50 µg/mL. The viability of amoebae was examined and compared with the treatment thiourea control and NaCl cytotoxicity control at the same concentration. The number of viable FLA was calculated from the first 50 FLA that observed using inverted microscope with 100x magnification. One-way ANOVA was used to statistically analyse the data and Fisher's Least Significant Difference (LSD) test was conducted to analyse the significant data. Results of amino acid-thiourea derivative treatment showed potential anti-amoebic activity against non-pathogenic FLA. Test model of anti-amoebic activity was established and further studies can be carried out by using the same compound at different concentration levels.

Keyword: Anti-amoebic activity, free living amoeba, amino acid-thiourea derivative, preliminary test method

ABSTRAK

Kajian ini telah dijalankan untuk mendapatkan data permulaan daripada ujian anti-amoebic yang menggunakan amoeba hidup bebas (FLA) yang bukan patogenik dan seterusnya merangkakan kaedah ujian awal yang sesuai untuk aktiviti anti-amoebic. Ujian anti-amoebic ini telah dilakukan dengan merawat amoeba hidup bebas dengan derivative asid amino -thiourea pada kepekatan 50 µg / mL. Kemandirian sel amoeba telah diperiksa berdasarkan perbandingan dengan rawatan kawalan thiourea dan kawalan sitotoksiti NaCl pada kepekatan yang sama. Bilangan sel amoeba yang masih aktif telah ditentukan dari 50 FLA yang pertama diperhatikan dengan menggunakan mikroskop songsang yang bermagnifikasi 100x. ANOVA telah digunakan untuk menganalisis data dan ujian Fisher LSD telah dijalankan untuk menganalisis data yang signifikan. Keputusan bagi rawatan menggunakan derivative asid amino-thiourea menunjukkan potensinya dalam anti-amoebic aktiviti terhadap sel amoeba yang tidak berpatogenik. Model ujian aktiviti anti-amoebic telah dibentuk dan kajian lanjutan dapat dijalankan dengan menggunakan kepakatan sebatian yang berbeza

Kata kunci: Anti-amoebic aktiviti, percuma hidup amoeba, asid amino-thiourea derivatif, kaedah ujian awal

1.0 Introduction

Amoebiasis has caused a large section of the world population to suffer. Amoebiasis, also known as amoebic dysentery is a common parasitic infection of the human gastrointestinal tract (Public Health Agency of Canada, 2005). It is caused by a protozoan parasite which known as *Entamoeba histolytica* and is the third leading parasitic cause of death in humans after malaria and schistosomiasis (Dhawan & Naparst, 2010). The World Health Organization (WHO) (1985) had reported that there was 480 million people were infected by *E. histolytica* globally in year 1981 and approximately 10% of infected people which is 48 million people suffer from invasive amoebiasis. Annually, it is responsible for 40, 000 to 100, 000 death cases in the world.

Amoebiasis is distributed world-wide and normally, people who live in China, Mexico, the eastern portion of South America, south-east and west Africa, and the whole of south-east Asia including the Indian subcontinent are in high risk to be infected by *E. histolytica* (WHO, 1985). Amoebiasis is present in tropical and subtropical developing areas and it is also a health problem in travelers, immigrants and male homosexuals in developed world (Tasawar *et al.*, 2010). Hence, the percentage of infected people with invasive amoebiasis is higher in the less developed countries where people live under poor economic and health conditions with insufficient sanitary facilities.

For instance, metronidazole is the drug of choice for treating invasive amoebiasis

in both adults and children, but it may not be efficient in eliminating parasite cysts in the intestine (Bansal *et al*., 2004; Gonzales *et al*., 2009). Besides that, a study which done by Bansal *et al*. in year 2006 had been reported that metronidazole resistance in *E. histolytica* and metronidazole resistant *E. histolytica* strains had been maintained indefinitely in medium. Besides, as mentioned by Lasserre (1979) and Sondhi *et al*. (2001), there was evidence showed tumorogenic activity of metronidazole relating to long period of time of oral administration in mice and rats. Although there are no documented cases of cancer related the use of metronidazole, the usage of metronidazole is still in the considerable stage.

Synthesis of novel thiourea compounds and various studies on their activities have been done world-wide. Researchers have shown that thiourea derivatives possess remarkable pharmacological properties (Manjula *et al.*, 2009; Liu *et al.*, 2010; Li *et al*., 2011). Studies on anti-amoebic activities have been done on pathogenic strains of amoeba (Akma Ibrahim *et al*., 2011). However, there is no appropriate test method has yet been developed for anti-amoebic activity using non-pathogenic free-living amoeba (FLA) species. Hence, this study aims to test the anti-amoebic potential of thiourea derivatives on the non-pathogenic FLA.

Recently, Department of Chemistry in Universiti Malaysia Sarawak has synthesized five amino acid-thiourea derivative compounds. This study therefore focuses on developing an appropriate test method for anti-amoebic activity. Amino acid-thiourea derivatives are used to carry out this study in order to test the particular

amoeba that is isolated from the lake in the Sarawak Golf Club.

The objective of this study is:

- To isolate non-pathogenic free living amoebae.
- To develop a preliminary test method for anti-amoebic activity of non-pathogenic free living amoebae.
- To determine anti-amoebic potential of amino acid-thiourea derivative.

2.0 Literature Review

2.1 Thiourea

Thiourea is an organic compound that is consisting of carbon, nitrogen, sulfur and hydrogen and this compound is also known as thiocarbamide or sulfourea (Wan Zullkiple, 2011). It has the molecular formula of CH_4N_2S and its molecular weight is 76.12g/mol. Figure 1 shows the structure of thiourea, which occurred as the mixture of two tautomers, $S=C(NH_2)_2$ (thiourea) and $HS=CNHNH_2$ (isothiourea) (Wan Zullkiple, 2011).

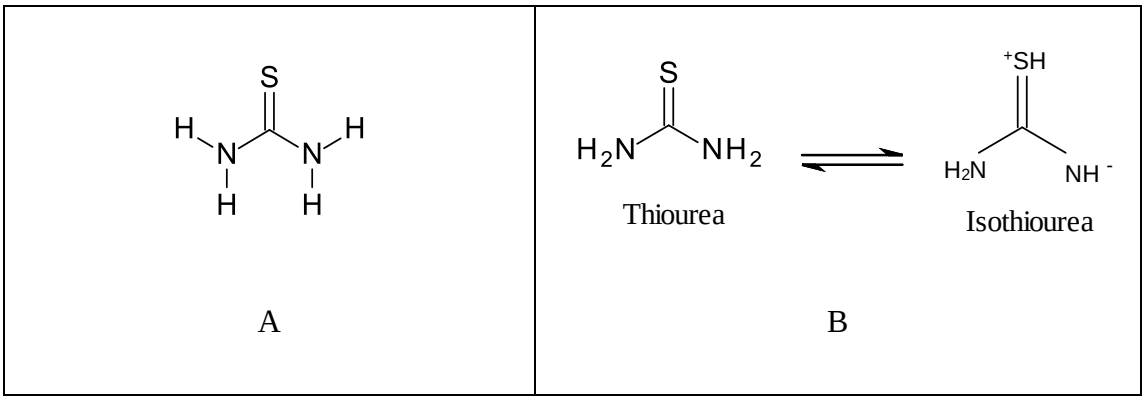
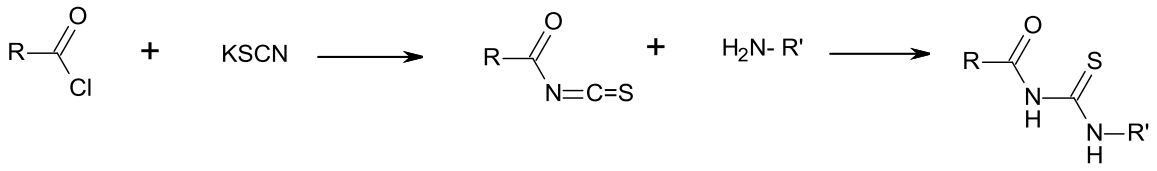


Figure 1: Structure of A: Thiourea compound, B: Tautomeric forms of thiourea.

Mohanta *et al*. (2000) stated that thiourea is an important compound that can be used as herbicides, pharmaceuticals, pesticides, rodenticides, vulcanization accelerator and as building agent in organic synthesis reaction. To synthesize thiourea, amino group is reacted with thiocyanate group in an appropriate solvent. These suitable solvent that are needed when synthesizing thiourea include ethanol (Cooke *et al.*, 2002) and tetrahydrofuran (THF) solution which is used by Liu *et al.* (2010) in his

study to produce a series of chiral thioureas bearing leucine and phosphate moieties. The general reaction is shown in Figure 2.



R= aryl or alkyl group

Figure 2: Scheme for the general reaction for synthesis of thiourea group.

2.1.1 Physical properties of thiourea

Thiourea compounds typically have a boiling point of 150℃ to 160℃ and melting point of 176℃ to 178℃. In general, thiourea exists as solid at room temperature and appears white in color. The compound has a density of 1.405g/cm^3. Thiourea is soluble in water and insoluble in non-polar solvent. Further, thiourea is also soluble in protic and aprotic organic solvents (WHO, 2003).

2.1.2 Amine groups in synthesis of thiourea derivatives.

Mohanta *et al.* (2000) had synthesized thiourea and its derivatives by using aniline, a primary amine group to synthesize symmetrical thiourea derivatives. o-pheylenediamine as shown in Figure 3 was also used to synthesise 2(1H)-benzimidazolinethione, a heterocyclic thiourea compound. It was reacted with 1-(methyldithiocarbonyl)imidazole, a transfer reagent, in ethanol reflux to give 2(1H)-benzimidazolinethione in high yield.

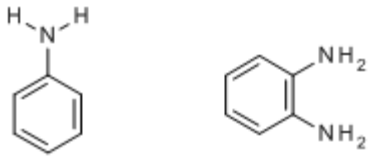


Figure 3: Primary amine compounds.

2.1.3 Thiol groups in synthesis of thiourea derivatives

Basically, a group having sulphur atom bonded to carbon atom is necessary in synthesizing of thiourea derivatives. Mohanta *et al.* (2000) had suggested that it is mainly introduced in the form of isothiocyanate or thiophosgene. For instance, Arslan *et al.* (2009) reported on the reaction of potassium thiocyanate with cyclohexanecarbonyl chloride to form thiocyanatocarbonyl compound. (*N*-(diethylcarbamothioyl)cyclohexanecarboxamide thiourea derivatives can be produced after thiocyanatocarbonyl compound undergoing further reaction with a series of secondary anime compounds. The molecular structure of potassium thiocyanate is indicated in Figure 4.

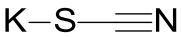
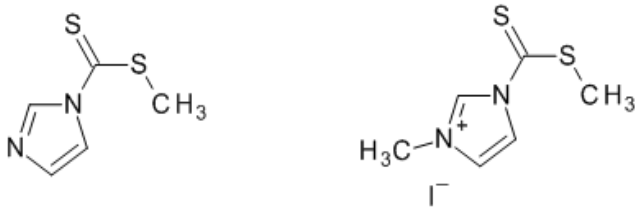


Figure 4: The structure of Potassium thiocyanate.

Instead of using isothiocyanate or thiophosgene in the reactions, Mohanta *et al.* (2000) had synthesised a useful thiocarbonyl transfer reagent and used it as a source of thiol group to produce substituted thiourea compounds. They synthesised 1-(methyldithiocarbonyl)imidazole (C) and its salt, 3-methyl-1-(methyldithiocarbonyl)

-imidazolium iodide (D). After that, amino acid was used to react with these compounds in order to produce thiourea derivatives, benzimidazoline-2-thione and imidazolidine-2-thione. Figure 5 indicates the thiocarbonyl transfer reagents that were used by Mohanta *et al.* (2000) in their study.



(C) (D)

Figure 5: The thiocarbonyl transfer reagents.

2.1.4 Pharmacological value of thiourea derivatives

Thiourea derivatives have been shown that to possess many biological activities, which are of pharmacological importance. These include antibacterial activity (Saeed *et al.*, 2009), antitumor property (Manjula *et al.*, 2009), antiviral activity (Kucukguzel *et al.*, 2008) and anticancer activity (Li *et al.*, 2011).

Besides possessing pharmaceutical properties, thiourea derivatives also can serve as ion-selective compound. A series of neutral bis thiourea ionophore which functioned as ion-selective electrodes was synthesised by Nishizawa *et al.* (1998). Thus, thiourea derivatives are widely applied in production of ion electrode and receptor. Based on these evidences, amino acid-thiourea derivatives have a very high level of pharmaceutical value.

2.2 Free-living Amoebae (FLA)

The phylum protozoa include many microorganisms which are related to human health and environment. As stated by Szenasi *et al.* (1998), protozoa causing human diseases can be divided into those that have a life cycle with vector-mediated transmission to the human host (e.g. *Plasmodium* and *Trypanosoma* spp.), those that are transmitted directly between human hosts (e.g. *E. histolytica*) and amphizoic small amoebae which cause infections of man (e.g. *Naegleria* and *Acanthamoeba*). Amoebae are complex, unicellular eukaryotic protists which possess clear protoplasm to form pseudopodia (Cuomo *et al.*, n.d.; Sherwin, 2009). According to Cuomo *et al.* (n.d.), amoebae use their pseudopodia for locomotion and engulf bacteria and red blood cells for feeding purposes. These FLA have the ability of surviving in a variety types of habitats and under a wide range of environmental conditions. The example of habitats that FLA can be found is soil, aquatic environment and dust (Szenasi *et al.*, 1998). Hence, water sources which are utilised for showering purposes may receive thermal discharges containing pathogenic free-living amoebae, and may become a dangerous source of infection for man. Nevertheless, the gut of the invertebrate and vertebrate hosts is the common habitat for those comensal and parasitic amoebae (Bush *et al.*, 2001).

2.2.1 Classification of free-living amoebae

The classification of free living amoebae can be divided into two types which are classified according taxa that are considered to be evolutionarily related (Adl *et al.*,

2005) and according to their phatogenicity (Bhatia & Ichhpujani, 2002). Protozoa was divided by the classical taxanomic classification into four groups which are Saecodina (amoebae), Mastigophora (Flagellates), Sporozoa (most parasitic protozoa) and Infusoria (ciliates) (Visvesvara *et al.*, 2007). However, based on modern morphological approaches, biochemical pathways, and molecular phylogenetics, the International Society of Protozoologists abandons this taxanomy classification (Adl *et al.*, 2005). The older hierarchical systems which consist of 'kingdom', 'phylum', 'class', 'subclass', 'super order', 'order' has been replaced by a new pattern of classification. As mentioned by Visvesvara *et al.* (2007), this new classification categorizes the Eukaryotes into six 'Super Group', namely Amoebozoa, Opisthokonta, Rhizaria, Archaeplastida, Chromalveolata and Excavata. The three genera which are going to discuss later are *Acanthamoeba, Entamoeba* and *Naegleria*. *Acanthamoeba* and *Entamoeba* are both classified under Super Group Amoebozoa, i.e. Amoebozoa: Acanthamoebidae and Amoebozoa: Entamoebidae (Visvesvara *et al.*, 2007; Sherwin, 2009). On the other hand, *Naegleria* is classified under Excavata: Heterolobosia: Vahlkampfiidae (Visvesvara *et al.*, 2007).

Apart from taxonomic classification, free-living amoebae also can be classified based on their pathogenicity (Bhatia, 2002). They can be divided into two major groups which are pathogenic amoebae and non-pathogenic amoebae. The free living amoebae that are categorized into pathogenic amoeba are those can cause parasitic diseases to their hosts (Visvesvara *et al.*, 2007). The examples of pathogenic amoebae are *Acanthamoeba* spp., *Balamuthia mandifloris*, *Naegleria fowleri* and *Sappinia*

diploidea (Sawyer, 1989; Zeybek *et al.*, 2010). *Acanthamoeba* spp. can cause human infectious including granulomatous amoebic encephalitis and amoebic keratitis (Rocha-Azevedo & Silva-Filho, 2007). In contrast, non-pathogenic amoebae refer to amoebae which serve as comensal in their hosts and do not cause infectious diseases to their hosts (Blessmann *et al.*, 2002). The example of non-pathogenic amoeba is *Entamoeba dispar* which does not associate with amoebic disease. Sometimes, it is hard to differentiate between pathogenic and non-pathogenic amoebae. This is because the morphology of some species of amoeba is difficult to be distinguished (Tasawar *et al.*, 2010). For example, *E. histolytica* can be confused with non-pathogenic amoeba like *E. moshkovskii* because they are morphologically indistinguishable. (Blessmann *et al.*, 2002; DiMiceli, 2004).

2.2.2 Genus *Entamoeba*

The genus *Entamoeba* consists of many species, six of which (*E. histolytica*, *E. coli*, *E. hartmanni*, *E. dispar*, *E. moshkovskii* and *E. polecki*) act as comensal in the human intestinal lumen (Sherwin, 2009; Tasawar *et al.*, 2010). Basically, most of them are non-pathogenic amoebae which reside in the human hosts, except for the *E. histolytica*. *E. histolytica* is classified under family Enatamoebae and is a tissue-lysing luminal protozoan parasite that is distributed world-wide (Sherwin, 2009). According to Cuomo *et al.*, the life cycle of *E. histolytica* includes 2 stages which are the infectious cyst stage and the pathogenic trophozoite stage (Figure 6). Its life cycle does not require any intermediate host which means that the amebic disease that is

associated with *E. histolytica* can be transmitted directly through human (Szenasi *et al.*, 1998).

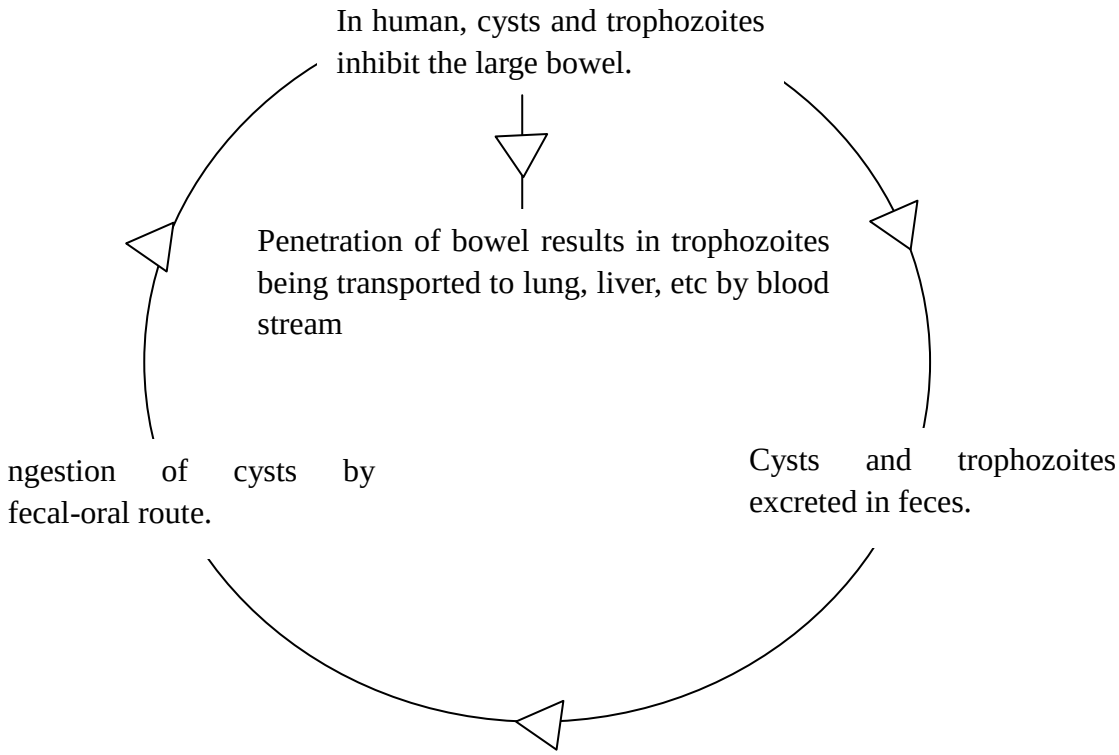


Figure 6: Life cycle of *Entamoeba histolytica*.

According to Dhawan and Naparst (2010), trophozoite of *E. histolytica* averages 25μm, ranging from 10 to 60μm and contains a single 3mm to 5mm nucleus with fine peripheral chromatin and a central nucleolus. Within the trophozoite, ingested red blood cells (RBC) may be present. However, the diameter of cysts of the *E. histolytica* is 10 to 15μm and there are one to four nuclei within the cysts. Meanwhile, the iodine-stainable glycogen clumps and chromatoid bodies with smooth rounded edges also can be found within the cysts (Cuomo *et al.*, n.d.). As mentioned by Sherwin (2009), this parasite possesses various mechanisms in order to enable the invasion of the host tissues. These mechanisms include contact-dependent cytolytic mechanisms

and biochemical mechanisms such as secretion of hydrolytic enzymes like proteinases, phosphatases and glycosidases. The life cycle for other non-pathogenic *Entamoeba* species are similar to the life cycle of *E. histolytica* but they do not have the invasive stage and do not feed on RBC (Cuomo *et al.*, n.d.).

2.3 Amoebiasis

As discussed above, amoebiasis is caused by the invasive luminal form of E. histolytica and this disease is distributed world-widely, especially to those tropical and subtropical developing countries (Thomas & Yap, 1986). Those areas with poor health and hygienic condition will have high prevalence in outbreak of this infectious disease (Tasawar *et al.*, 2010). Due to humans are the natural reservoirs of the parasite, so the transmission of this disease is mainly depending on the carriers who are excreting the cysts (Public Health Agency of Canada, 2005). Usually, it is transmitted through fecal-oral route, when the infected people ingested the contaminated food or water (Dhawan & Naparst, 2010).

Normally, the protozoa survive in the large intestine without causing noticeable diseases. However, certain virulence strains of amoeba become invasive and penetrate into the intestine or other extra-intestinal tissue damaging the tissue and causing the chronic amoebic disease (Thomas & Yap, 1986). WHO (1985) had stated that the main forms of invasive amoeba are amoeba dysentery and liver abscess reflect the parasites and host factors which result in pathological lesions of amoebic colitis. Diffusion of the organism to extra-colonic sites, mostly the liver is due to the invasion

of the colonic mucosa (Dhawan & Naparst, 2010).

2.4 Anti-amoebic drugs

In year 1912, Emetine, an active alkaloid of ipecacuanha plant was introduced for the treatment of amoebiasis and it is the standardized drug for treating this disease for the next 50 years (Lasserre, 1979). However, its usefulness was hindered by the toxicity. For instance, the most common anti-amoebic drug for the treatment of invasive amoebic disease is nitroimidazole, which consists of metronidazole and tinidazole (Bansal *et al.*, 2004). Besides that, drugs like halogenated hydroxyquinolines, arsenicals and diloxanide furoate are also used to treat amoebiasis but all of them are somehow show the inadequately in the efficiency in treating the disease (Lasserre, 1979). For the moment, there is no single-dose treatment available that can yet be implemented.

3.0 Materials and Methods

3.1 Preliminary amoeba culturing

3.1.1 Water samples collection

Water samples were collected from the lakes which are located in the Sarawak Golf Club (Figure 7 & 8). All material and apparatus, including Schott bottle, 70% ethanol, gloves were prepared on the day before. Aseptic technique was followed when preparing all materials and apparatus in order to reduce microbial contamination. Water sample were collected using 250 ml Schott bottle. The bottle was dipped and inverted under the surface of the lake until it reached 5 to 7 cm above the sediment of the lake. The bottle was slanted in order to facilitate the water sample collection. Water samples were collected at the side of the two different lakes which are named Lake I (Figure 7) and Lake II (Figure 8).

3.1.2 Water sample amoebae observation

The water samples were taken with three replicates for each lake. All water samples were observed under light microscope for the presence of amoeba by transferring 50µl of the water sample onto slide and gently adding a coverslip on the transferred water sample.

Figure 7: Lake I which is located in the Sarawak Golf Club.

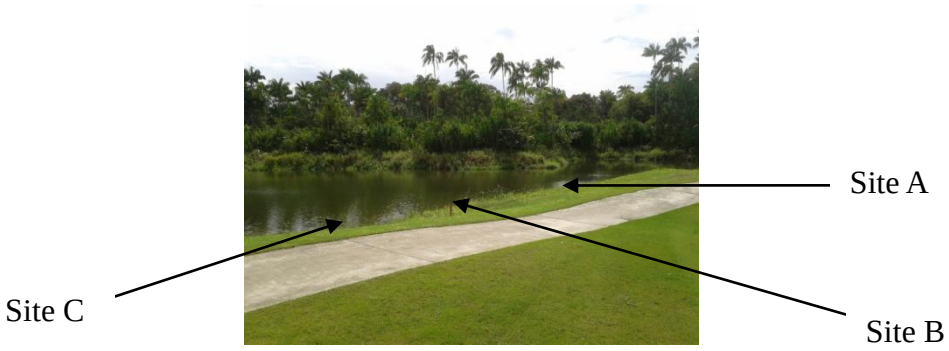


Figure 8: Lake II which is located in Sarawak Golf Club.

3.1.3 Initial culturing of amoebae

For amoeba culturing, standard aseptic techniques were applied throughout the study. When the water samples were transferred to the laboratory, sterile rice grains with 2.6g and 5.1g were added into the water sample from Lake II in order to enrich the amoeba population before conducting the process of isolation (Kirby, 1950). The water sample which did not contain rice grains was acted as control. After adding the rice grains, the water samples were examined under microscope after 1, 3 and 5 days incubation in the Schott bottle with five different fields per slide to ensure detection of the presence of amoebae. Aliquot of 50µl of water sample were transferred from respective water sample onto the slide and viewed under the light microscope at 100× magnification. The number of amoebae that were observed from five different fields

was recorded.

3.1.4 Preparation of media and apparatus

All the apparatus that were used for media preparation and heat-inactivation *E. coli* testing were sterilised by using autoclave machine. These apparatus include Schott bottles, universal bottles, bijou bottles, pipette tips, filter paper and funnel. For surface sterilisation, 70% ethanol was prepared and applied. The nutrient broth (NB) and nutrient (NA) were prepared by weighing appropriate agar powder and mixed with exact volume of distilled water. All media were stirred by using magnetic stirrer and were sterilized by using autoclave machine at 15 psi, 121℃ for 15 minutes prior to use. The NA was transferred from Schott bottle into Petri dishes in laminar flow hood and was kept in the refrigerator at 4 ℃ for future use.

3.1.5 Preparation of heat-inactivated *Escherichia coli*

Heat-inactivated *E. coli* was prepared from the freshly grown cultures. Firstly, 40ml of NB was transferred from Schott bottle into universal bottles. Then, 5µl of *E. coli* from the E. coli glycerol stock was cultured in the NB and incubated in the incubator shaker for 24 hours. The grown *E. coli* was sub-cultured from the NB into the NA plates in order to obtain pure *E. coli* culture. After one day incubation, the NA plates were observed and identification of *E. coli* culture was carried out by Gram staining.

Once the culture was identified as pure *E. coli* culture, a 2ml of aliquots was transferred into bijou bottles. The *E. coli* cultures were then subjected to heat