Coptosapelta flavescens **as a Potential Anti-*Entamoeba histolytica* and Anti-*Giardia intestinalis***

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A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Candidate

ชื่อวิทยานิพนธ์	ศักยภาพของย่านขลง (*Coptosapelta flavescens*) ในการต้านเชื้อ *Entamoeba histolytica* และ *Giardia intestinalis*
ผู้เขียน	นางสาวเครือวัลย์ หวนกัง
สาขาวิชา	จุลชีววิทยา
ปีการศึกษา	2557

บทคัดย่อ

ทำการทดสอบสารสกัด 23 สารสกัดที่สกัดจากสมุนไพรไทยมีฤทธิ์แก้ท้องร่วงหรือขับพยาธิ 21 ชนิด ต่อการเจริญของเชื้อโปรโตซัวในลำไส้ชนิด *Entamoeba histolytica* และ *Giardia intestinalis* จากการทดลอง พบว่าสารสกัดอะซิโตนจากย่านขลง (*Coptosapelta flavescens*) ให้ผลการยับยั้งเชื้อ *E. histolytica* และ *G. intestinalis* ดีที่สุดด้วยค่า Minimal Inhibitory Concentration (MIC) เท่ากับ 125 และ 15.63 µg/ml ตามลำดับ สารสกัดจากขมิ้น (*Curcuma longa*), น้ำนมราชสีห์เล็ก (*Euphorbia thymifolia*), มังคุด (*Garcinia mangostana*), ทับทิม (*Punica granatum*), กระท้อน (*Sandoricum koetjape*) และ สมอภิเภก (*Terminalia bellerica*) ยับยั้งเชื้อโปรโตซัวทั้งสองชนิดได้ปานกลาง (MIC 250-500 µg/ml) สารสกัดจากเถาวัลย์เปรียง (*Derris scandens*) ยับยั้งได้เฉพาะเชื้อ *E. histolytica* ด้วยค่า MIC 500 µg/ml สารสกัดจากพลู (*Piper betle*), ฝรั่ง (*Psidium guajava*), โกงกาง (*Rhizophora mucronata*) และ สมอไทย (*Terminalia chebula*) ยับยั้งได้เฉพาะเชื้อ *G. intestinalis* ด้วยค่า MIC 250-500 µg/ml สำหรับยา metronidazole ซึ่งเป็นยามาตรฐานยับยั้งเชื้อทั้งสองสายพันธุ์ด้วยค่า MIC เท่ากับ 2.5 µg/ml

นำสารบริสุทธิ์ 3 สาร คือ anthraquinones 2 สารและ naphthoquinone 1 สาร ที่แยกได้จากย่านขลงมาทดสอบกับเชื้อโปรโตซัวทั้งสองสายพันธุ์ พบว่า สารบริสุทธิ์ 1-hydroxy-2-hydroxymethylanthraquinone (CFQ) ให้ค่าการยับยั้งดีที่สุดทั้งกับเชื้อ *E. histolytica* และ *G. intestinalis* ด้วยค่า MIC เท่ากับ 20 และ 2.5 µg/ml ตามลำดับ ค่า IC_{50} ของยา metronidazole และสาร CFQ ต่อเชื้อ *E. histolytica* เท่ากับ 0.44 µg/ml และ 4.59 µg/ml ตามลำดับ ขณะที่ค่า IC_{50} ของยา metronidazole และสาร CFQ ต่อเชื้อ *G. intestinalis* มีค่าเท่ากันคือ 0.42 µg/ml ใน time killing assay พบว่า เปอร์เซ็นต์การรอดชีวิตของ *G. intestinalis*

หลังจากทดสอบด้วยสาร CFQ ต่ำกว่าการทดสอบกับยา metronidazole อย่างมีนัยสำคัญ ($p<0.05$) เมื่อทำการบ่มเชื้อที่เวลา 6 และ 12 ชั่วโมง ในขณะที่เมื่อทดสอบสาร CFQ กับเชื้อ *E. histolytica* พบว่าอัตราการรอดชีวิตของเชื้อ *E. histolytica* ใกล้เคียงกับการทดสอบกับยา metronidazole

จากการวิเคราะห์ลักษณะโครงสร้างของเชื้อ *E. histolytica* และ *G. intestinalis* ด้วย SEM และ TEM หลังจากทดสอบด้วยยา metronidazole และสาร CFQ ที่ความเข้มข้นเท่ากับค่า IC_{50} เป็นเวลา 24 ชั่วโมง พบว่า ทั้งยา metronidazole และสาร CFQ มีฤทธิ์ทำให้ *E. histolytica* มีผนังเซลล์ที่ขรุขระ และเกิดรูที่เยื่อหุ้มเซลล์ สำหรับใน *G. intestinalis* ทั้งยา metronidazole และสาร CFQ ทำให้เซลล์เหี่ยวและมีลักษณะกลม เยื่อหุ้มเซลล์โป่งพอง ventral disc ถูกทำลาย เกิดการตกตะกอนของ electron ในนิวเคลียส อย่างไรก็ตามการแตกสลายของเยื่อหุ้มเซลล์พบเฉพาะในเชื้อ *G. intestinalis* ที่ทดสอบกับสาร CFQ เท่านั้น

เนื่องจากค่า IC_{50} ของสาร CFQ ต่อการยับยั้งการเจริญของเชื้อ *E. histolytica* มีค่าค่อนข้างสูง (4.59 µg/ml) เมื่อเทียบกับยา metronidazole (0.44 µg/ml) จึงได้ศึกษาเพิ่มเติมเฉพาะกลไกของสาร CFQ ต่อการต้านเชื้อ *G. intestinalis* เท่านั้น เมื่อทำการประเมินว่าฤทธิ์ของสาร CFQ มีผลให้ *G. intestinalis* เกิด apoptosis หรือไม่ โดยใช้การย้อมเชื้อด้วย AnnexinV-FITC แล้ววิเคราะห์ด้วยเครื่องโฟลไซโตเมทรี พบว่า สาร CFQ (IC_{50}) ทำให้ *G. intestinalis* ในระยะโทรโฟซอยท์เกิด apoptosis ตั้งแต่การบ่มที่ 6 ชั่วโมง ในขณะที่ยา metronidazole ทำให้เกิด apoptosis ได้เพียงเล็กน้อย ในขณะที่ ส่วนใหญ่ *G. intestinalis* (ร้อยละ 74.8) อยู่ในระยะ late apoptotic cell หลังจากทดสอบกับสาร CFQ ที่เวลา 24 ชั่วโมง เมื่อตรวจสอบการแตกหักของ DNA ด้วยวิธี TUNEL ก็ยืนยันการเกิด late apoptosis เช่นกันเมื่อวิเคราะห์วงจรชีวิตของเชื้อ หลังจากทดสอบด้วยยา metronidazole และสาร CFQ พบว่า การกระจายของปริมาณ DNA ในแต่ละระยะ ไม่มีความแตกต่างจากกลุ่มควบคุม แต่ยา metronidazole และสาร CFQ ที่ความเข้มข้นเท่ากับค่า IC_{50} สามารถลดการเกาะติดของเชื้อ *G. intestinalis* ต่อเซลล์ Caco-2 อย่างมีนัยสำคัญตั้งแต่การบ่มที่เวลา 6 ชั่วโมง จากผลการทดลองทั้งหมดสามารถอธิบายกลไกของสาร CFQ ต่อเชื้อ *G. intestinalis* และใช้เป็นข้อมูลสำคัญในการที่จะพัฒนาการใช้ CFQ ในการรักษาโรคติดเชื้อที่เกิดจาก *G. intestinalis*

Thesis Title   *Coptosapelta flavescens* as a potential anti-*Entamoeba histolytica* and anti-*Giardia intetinalis*

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ABSTRACT

Twenty-three extracts from 21 Thai medicinal plants which were claimed to possess anti-diarrhoeal or anti-parasitic activity, were screened for their activity against *Entamoeba histolytica* and *Giardia intestinalis*. An acetone extract from *Coptosapelta flavescens* was most active against both *E. histolytica* and *G. intestinalis* with minimal inhibitory concentration (MIC) of 125 and 15.63 µg/ml, respectively. The extracts from *Curcuma longa*, *Euphorbia thymifolia*, *Garcinia mangostana*, *Punica granatum*, *Sandoricum koetjape* and *Terminalia bellerica* exhibited moderate activity against both protozoa (MICs 250-500 µg/ml). *Derris scandens* extract effect only *E. histolytica* at MIC of 500 µg/ml, and the extracts from *Piper betle*, *Psidium guajava*, *Rhizophora mucronata* and *Terminalia chebula* showed inhibition activity against *G. intestinalis* only with MICs of 250-500 µg/ml. MIC of metronidazole, a standard drug, against both protozoa was 2.5 µg/ml.

Three compounds, two anthraquinones and one naphthoquinone were obtained from *C. flavescens*. It was found that 1-hydroxy-2-hydroxymethylanthraquinone (CFQ) was the most active chemical against *E. histolytica* and *G. intestinalis* with MIC values of 20 and 2.5 µg/ml, respectively. IC_{50} of metronidazole and CFQ against *E. histolytica* was 0.44 and 4.59 µg/ml, respectively, whereas IC_{50} of both metronidazole and CFQ against *G. intestinalis* was 0.42 µg/ml. In time killing assay, the percentage of viable *G. intestinalis* after exposed to CFQ showed significantly ($p<0.05$) lower than trophozoite exposed to a standard drug, metronidazole, at 6 and 12 h of incubation time, while its activity against *E. histolytica* was comparable to metronidazole.

SEM and TEM analyses of *E. histolytica* and *G. intestinalis* after incubated with IC_{50} concentration of metronidazole and CFQ at 24 h indicated that both metronidazole and CFQ induced several physical alterations including rough cell membrane with a number of holes on the membrane of *E. histolytica*. In *G. intestinalis*, both metronidazole and CFQ caused the appearance of wrinkled and rounded cells, membrane blebbing, ventral disc damage and electron dense precipitates in the nuclei. However, membrane rupture was found only in *G. intestinalis* exposed to CFQ.

As the IC_{50} value of CFQ against *E. histolytica* was considerably high value (4.59 µg/ml) when compared to metronidazole (0.44 µg/ml). We therefore further studied focusing on the mechanism of CFQ against *G. intestinalis* only. We assessed whether CFQ was an inducer of apoptosis by the AnnexinV-FITC assay, and viewed by flow cytometry. CFQ at its IC_{50} value induced apoptosis as early as 6 h after incubation, whereas metronidazole produced little or no apoptosis at its IC_{50} value. At 24 h incubation most (74.8%) *G. intestinalis* trophozoite exposed to CFQ underwent late apoptotic cell. TUNEL assay at 24 h incubation confirmed the finding that most *G. intestinalis* trophozoite after exposed to CFQ were in late apoptosis as most *G. intestinalis* trophozoite presented with condensed and fragmented orange chromatin. Cell cycle analysis showed that the distribution of DNA through cell cycle of *G. intestinalis* treated with metronidazole and CFQ were not significantly different from the control without drugs, indicating that both drug had no affect on *G. intestinalis* cell cycle. On the other hand, both metronidazole and CFQ at IC_{50} concentration were significantly detached *G. intestinalis* trophozoites from Caco-2 cell line when compared to control since 6 h incubation. Taken together, we have provided a mechanistic explanation of the action of CFQ against *G. intestinalis* trophozoites. These results have provided further evidence that CFQ could be a new potential compound for use against *G. intestinalis*.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	Degree Celsius
mg	=	Milligram
µg	=	Microgram
ml	=	Milliliter
nm	=	Nanometer
µl	=	Microliter
µm	=	Micrometer
%	=	Percentage
NaOH	=	Sodium hydroxide
HCl	=	Hydrochloric acid
DMSO	=	Dimethyl sulfoxide
g	=	Gram
g	=	Gravitational force
MIC	=	Minimal inhibitory concentration
SD	=	Standard deviation
SEM	=	Standard error of mean
MEM	=	Minimal Essential Medium

CHAPTER 1

INTRODUCTION

1.1   Background and rationale

Intestinal infection caused by parasitic protozoa is one of the main public health problems in the developing countries (Calzada et al., 2005; Amaral et al., 2006; Brandelli et al., 2009). *Entamoeba histolytica* and *Giardia intestinalis* (Syn. *G. lamblia* or *G. duodenalis*) are intestinal protozoa commonly found in human worldwide (Adam, 2001; Verweij et al., 2004; Calzada et al., 2005; Brandelli et al., 2009). *E. histolytica* causes amoebic dysentery with a death rate of over 100,000 annually, the third cause of mortality by parasitic infection after malaria and schistosomiasis (Zlobl, 2001). *G. intestinali*s, a flagellate protozoan found in small intestine causes chronic diarrhea and growth retardation in children in developing countries (Perez-Arriaga et al., 2006; Machado et al., 2010). It is estimated that about 300 million people, particularly children were infected per year (Brandelli et al., 2009).

Metronidazole is the most common drug used for the treatment of intestinal infections caused by *E. histolytica* or *G. intestinalis* (Adam, 2001; Moundipa et al., 2005), but unpleasant side effects such as metallic taste, headache, nausea, urticaria, pruritus and dark colored urine have been reported (Calzada et al., 2006; Vidal et al., 2007). Furthermore, metronidazole is mutagenic in bacteria (Gardner and Hill, 2001) and drug resistance of the parasite was found (Upcroft and Upcroft, 2001; Jimenez-Cardoso et al., 2004). Therefore, continued search for new anti-protozoal compounds with high activity, safe and little or no side effects is still a necessary goal.

The use of medicinal plants in developing countries is popular because their products are safe, widely available at low cost and easy to access. Moreover, most Thai people with diarrhea tend to first seek help from traditional healers

dispensing traditional Thai medicines. Several compounds from herbs have been proved to be effective against various diseases. Curcumin from *Curcuma longa* has reported to possess anti-bacterial, anti-oxidant, anti-HIV and anti-protozoal activities (Rasmussen et al., 2000; Chattopadhyay et al., 2004). Essential oils from *Thymbra capitata*, *Origanum virens*, *Thymus zygis* L. subsp. *sylvestris*, *Lippia graveolens* and *Syzygium aromaticum* are active against *G. intestinalis* (Perez-Arriaga et al., 2006; Machado et al., 2010). In addition, essential oils from *Origanum vulgare* and *Pulicaria odora* demonstrated anti-bacterial activity against some pathogenic bacteria such as *Bacillus cereus*, *Streptococcus* sp., and *Escherichia coli* (Bakkali et al., 2008). Thus, considering the bioactive potential of medicinal plants, in this present study, it was attempted to evaluate *in vitro E. histolytica* and *G. intestinalis* activity of selected medicinal plants in order to search for an alternative drug suitable for use in preventing and treating infections caused by *E. histolytica* and *G. intestinalis*.

1.2 Review of literature

1.2.1 *Entamoeba histolytica*

Amoebiasis was first described by Fedor Losch from a young farmer who admitted to his clinic in Saint Petersburg, Russia with dysentery in 1875. The amoeba which he believed to be a causative agent was isolated and named *Amoeba coli* (Stilwell, 1955; Ackers, 1996). In 1890, Sir William Osler reported the first case of amoebiasis from North American when he observed amoeba in stool and abscess fluid from a physician who previously resided in Panama. In the same year, Henri Lafleur, a Canadian resident at Johns Hopkins, reported a case of dysentery in a sailor from whom *Amoeba coli* was isolated in the stool. Moreover, the cyst form of amoeba was first demonstrated by Quincke and Roos in 1893 (Imperato, 1981). *E. histolytica* was named by Fritz Schaudinn in 1903. The next major breakthrough came with the work in 1913 of Walker and Shellard who demonstrated *E. coli* was nonpathogenic and *E. histolytica* was pathogenic. However, in 1925 Emile Brumpt, the French parasitologist, described another amoeba with quadrinucleate cysts, which he named

E. dispar. Consequently, the name *E. histolytica* was set for the invasive and pathogenic amoeba and *E. dispar* was set for the noninvasive amoeba in accordance with Brumpt's original hypothesis from 1925 (Diamond and Clark, 1993). In 1997, the World Health Organization defined amoebiasis as infection with the protozoan parasite *E. histolytica* (WHO, 1997a).

1.2.1.1 Morphology

E. histolytica occurs in humans in two forms: trophozoites and cysts. Trophozoites, actively motile by finger-like pseudopodia, are about 10 to 60 μm in diameter. The cysts are round structures around 10 to 16 μm in diameter (Figure 1). Immature cysts contain one or two nuclei, whereas, the mature cyst containing four nuclei and it is the infective stage.
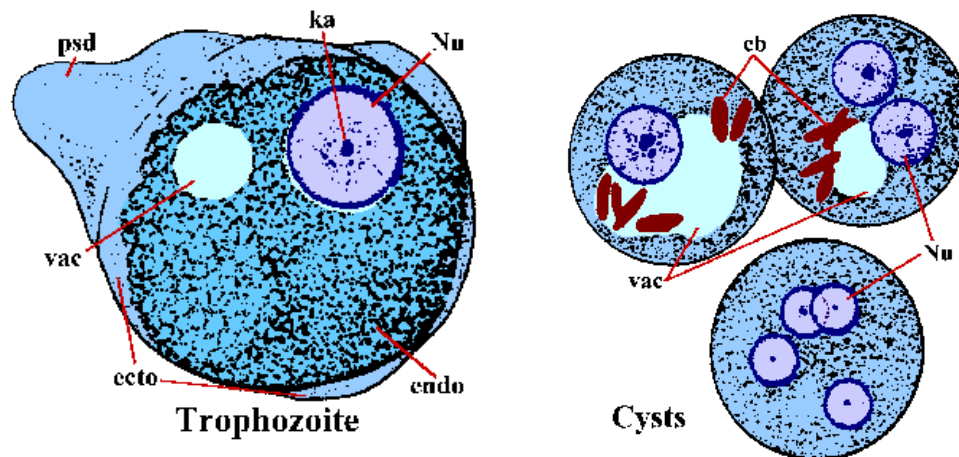
Figure 1 Morphology of *Entamoeba histolytica* in term of trophozoites and cysts

(Source: http://www.tulane.edu/~wiser/protozoology/notes/intes.html)

1.2.1.2   Life cycle

Human infection usually begins with the ingestion of the cyst in fecal contaminated food or water. The ingested cyst passes through the stomach into the small intestine where excystation occurs resulting in the emergence of a 4 nucleated metacyst and finally divided into eight trophozoites. These trophozoites migrate to and multiply in the colon. Occasionally, trophozoites attack and invade the intestinal mucosa causing dysentery and/or progress through the blood vessels to extraintestinal locations such as liver, lungs and brain, where they may form life-threatening abscesses. Under the initiation of unfavorable condition, a cystic wall starts to develop in each trophozoite that is encystation called pre-cystic stage. The single nucleus of the cyst soon undergoes a division to form two and then into four daughter nuclei. These quadrinucleated cysts do not develop further in the large intestine. Both trophozoites and cysts are then expelled with the feces into the environment. Cysts can survive for prolonged periods outside the host while the trophozoites survive only for a few hours (Sehgal et al., 1996) as shown in Figure 2.
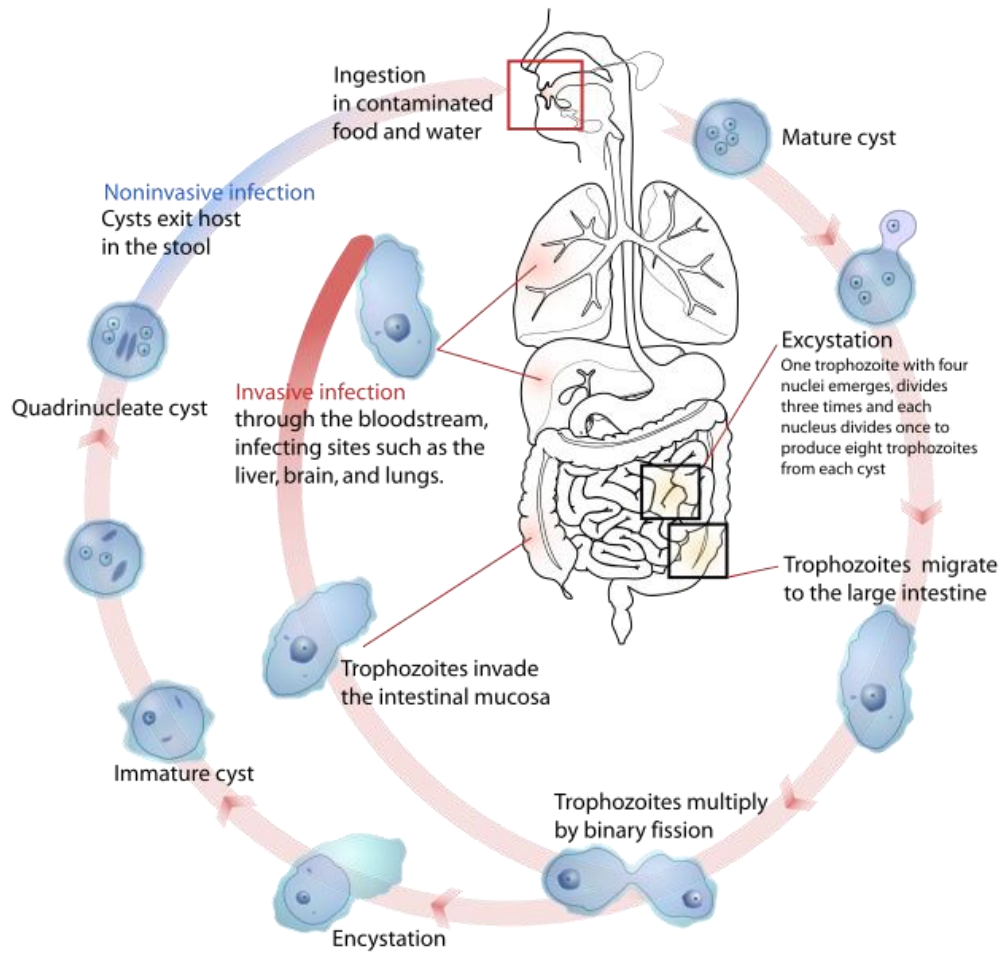
Figure 2 Life cycle of *Entamoeba histolytica*

(Source: http://en.wikipedia.org/wiki/Entamoeba_histolytica#mediaviewer/

File:Entamoeba_histolytica_life_cycle-en.svg)

1.2.1.3 Pathogenicity and virulence

The severity of intestinal and extraintestinal amoebiasis mainly based on the site of infection. Invasion of the mucosa probably depends on the pathogenicity of *E. histolytica* strain, host resistance and other factors such as, parasite motility, pro-inflammatory and protease host immune reaction (Juniper, 1984; Ralston and Petri, 2011). It is believed that pathogenesis of *E. histolytica* infection depends on three virulence factors as follows:

- Gal-GalNAc lectin. The adherence of *E. histolytica* trophozoite to the host target cells is mediated by the galactose or N-acetyl-D-galactosamine (Gal/GalNAc)-specific lectin, which binds to exposed terminal Gal-GalNAc residues present in target cells glycoproteins (Ravdin et al., 1989; Stanley, 2003; Ximenez et al., 2010).

- Amoebapores. A pore-forming protein complex is inserted into the host cell by the trophozoite, a rapid cytolytic event takes place that results in swelling, surface blabbing, and lysis of the inadvertent target cell, including lymphocytes, polymorphonuclear leukocytes, and macrophages, leaving the parasite unharmed (Bracha et al., 2002; Stauffer and Ravdin, 2003).

- Cytoproteins (cysteine proteases). Cysteine proteases are known to be important to the pathogenesis of *E. histolytica* and function by degrading extracellular matrix components, basement membrane proteins and through the disruption of cell monolayers. It has long been considered to play a central role in tissue invasion by *E. histolytica*. These were considered to be responsible for cell lysis and deep-flask shaped ulcers. In some cases, cysteine proteases induce the trophozoites to enter the circulation and reaching internal organs like the liver, lungs, skin, etc. (Keene et al., 1986; Scholze and Schulte, 1988; Que and Reed, 2000).

1.2.1.4 Clinical features

Most people infected with *E. histolytica* are defined as the presence of *E. histolytica* in stool in the absence of colitis or extraintestinal infection and represent "non-invasive" disease (Stanley, 2003). Generally, only a small proportion of people infected with *E. histolytica* will go on to develop clinical disease. Asymptomatic patients may excrete cysts for a short period of time and the infection will clear within 12 months. The most frequent manifestations are amoebic colitis and amoebic liver abscess (Stanley, 2003). Patients with amoebic colitis typically present with a several week history of gradual onset of abdominal pain and tenderness, diarrhea with visible blood and mucus in stools. The presence of haematophagous trophozoites (trophozoites with ingested red blood cells) in stools or tissues has been found (Adams and MacLeod, 1977). Moreover, the clinical symptoms associated with extraintestinal amoebiasis will depend on the affected organ like the liver, lungs, skin, etc. The most common extraintestinal site affected by the parasite is the liver and an amoebic liver abscess (Petri and Singh, 1999). In addition, pulmonary amoebiasis generally results from the direct extension of the liver abscess through the diaphragm and cutaneous amoebiasis is the result of skin or mucous membranes being bathed in fluids containing trophozoites.

1.2.1.5 Epidemiology

It is estimated that 500 million people worldwide are infected with *E. histolytica* and is responsible for 40,000-100,000 deaths each year (Zlobl, 2001; Stanley, 2003). Amoebiasis is still a main problem of human in developing countries with poor sanitation, especially in children of five years and below (WHO, 1997b; van Hal et al., 2007). Amoebiasis cases were found approximately 10% of the world's population and only 1% of infected individuals developed the invasive form of disease. Most amoebic infections cases occur in areas of Central and South America, Africa, and Asia (Petri and Singh, 1999). In Egypt, 38% of individuals presenting with acute diarrhea at outpatient clinic were found to have amoebic colitis (Stanley,

2003). Seroprevalence studies in South Africa and Egypt revealed 10 to 20% of the populations were positive against *E. histolytica* (Stauffer et al., 2006). The higher infection rates of *E. histolytica* infection have been observed in homosexual men from several countries (Peters et al., 1986; Ohnishi et al., 2003; Tsai et al., 2006). Moreover, tourists and immigrants who travel from the endemic area (Krogstad et al., 1978), HIV-positive individuals (Lowther et al., 2000), the handicapped (Lee et al., 2000) and institutionalized persons (Cheng and Wang, 1999) have been noticed to have high *E. histolytica* infection.

1.2.2 *Giardia intestinalis***:** Syn. *Giardia lamblia*, *Giardia duodenalis* (Homan and Mank, 2001)

Giardia intestinalis was first identified by Prof. Antony van Leeuwenhoek in 1681 when he examined his own diarrhea stools under microscope. In 1859 Lambl described the genus *Giardia* in greater detail. This species was named after two scientists who had studied it: Prof. A. Giard in Paris and Dr. F. Lambl in Prague in 1915 (Adam, 2001). In 1952, Filice published a detailed morphologic description of *Giardia* and divided this genus into three species: *G. duodenalis, G. muris*, and *G. agilis* on the basis of the morphology of the median body (Filice, 1952).

1.2.2.1 Morphology

G. intestinalis has two forms: the trophozoite and the cyst. Trophozoite is pear shape approximately 10 to 12 μm long by 5 to 7 μm wide with two nuclei, four pairs of flagella and ventral disk, a concave structure with a raised ridge at its anterior end, for attachment to the intestinal microvillus surface of the host.

The cyst, about 8 to 12 μm long by 7 to 10 μm wide, is surrounded by a 0.3 μm thick wall. The mature cyst containing four nuclei is the infective stage. The cysts resist to chlorine and can survive in cool water for weeks (Wolfe, 1992) as shown in Figure 3.

Figure 3 Morphology of *Giardia intestinalis* in term of trophozoites and cysts

(Source: http://aparasiteworld.blogspot.com/2010/01/giardia.html)

1.2.2.2 Life cycle

Transmission of *G. intestinalis* occurs most commonly by ingestion of cyst contaminated in water, food or fecal-oral contact (Adam, 1991; Wolfe, 1992; Ford, 2005). The cysts are passed through intestine and excystation occurs in the duodenum. Two trophozoites from each mature cyst establish themselves among the intestinal villi and replicate by binary fission. The cysts are formed in the jejunum and passed with feces into the environment (Lebwohl et al., 2003) as shown in Figure 4.
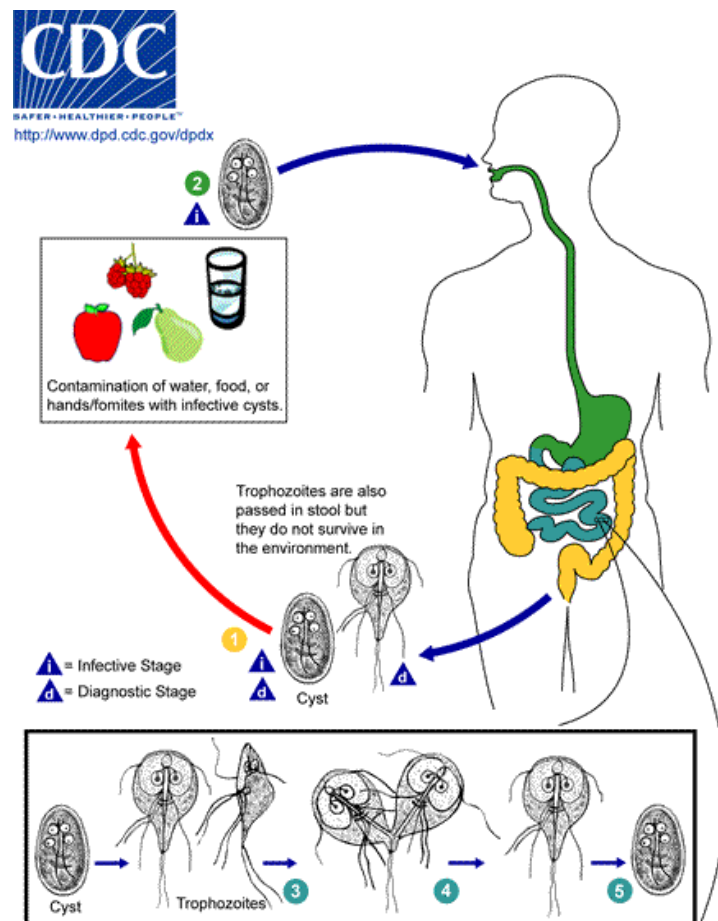
Figure 4 Life cycle of *Giardia intestinalis*

(Source: http://www.cdc.gov/parasites/giardia/biology.html)

1.2.2.3 Pathogenicity and virulence

The mechanism by which *Giardia* causes diarrhea and intestinal malabsorption has not been fully elucidated. Several hypotheses have been proposed. Ali and Hill (2003) showed that direct damage to the intestinal brush border and mucosa either by trophozoites or by host immune-response caused secretion of fluid leading to gut and duodenal flora alteration that contributes to diarrhea. An additional hypothesis is that *G. intestinalis* can induce apoptosis in infected cells (Eckmann et al., 2000; Chin et al., 2002). Furthermore, one or more of the following factors is believed to be involved (Buret, 2007):

- The expression of glycoprotein located on the surface of *G. intestinalis* trophozoites has been demonstrated to induce fluid accumulation in the intestine that caused *Giardia* release a variety of potentially ''toxic'' substances, such as proteinases and lectins that may be responsible for direct epithelial injury (Chen et al., 1995; Kaur et al., 2001; Sousa et al., 2001).

- *Giardia* attached to intestine caused the loss of intestinal brush border surface area and the corresponding decrease in disaccharidase enzymes activity leading to increase quantities of disaccharides in the intestinal lumen, which can result in osmotic diarrhea (Nain et al., 1991).

- *Giardia* infection in gerbils accelerates intestinal transit time and increases smooth muscle contractility, both of which may play a role in pathogenesis of giardial diarrhea (Eckmann and Gillin, 2001).

1.2.2.4 Clinical features

The signs and symptoms of giardiasis occur after ingestion as few as ten cysts and the incubation period is generally 9-15 days (Wolfe, 1992). The symptom can be characterized by gastrointestinal disorders, such as diarrhea, malabsorption, weight loss, nausea, vomiting and fatigue which may persist until six

weeks (Adam, 1991). An acute giardiasis, mainly seen in travelers, is eventually become chronic diarrhea.

1.2.2.5   Epidemiology

Giardia intestinalis is found worldwide especially in tropical and subtropical region with higher prevalence where sanitation is poor. The prevalence of giardiasis occurs in developing countries approximately 20% when compared with 5% in developed countries (Roxstrom-Lindquist et al., 2006). Furthermore, it has been estimated that about 200 million people are infected each year in Africa, Asia and Latin America (Norhayati et al., 2003). *Giardia* infection was estimated 2.5 million cases per year in the United States (Furness et al., 2000) and 0.9-6.15% in Italy (Giangaspero et al., 2007). It is a major cause of epidemic childhood and traveler's diarrhea where tap water is the primary source of drinking water (Welch, 2000; Yoder et al., 2008). Children under 10 years old who attend day care are especially at risk, with average infection rates of 2-20% (Arbo et al., 2006). In Thailand, the prevalence rate ranged from 1.25% to 37.7% (Dib et al., 2008) and incidence among Thai orphans is 37.7-85.5% (Saksirisampant et al., 2003).

1.2.3   Treatment of amoebiasis and giardiasis

Metronidazole is the first drug of choice recommended by the World Health Organization (WHO) for amoebiasis and giardiasis treatment. Patients with confirmed *E. histolytica* or *G. intestinalis* infections, even if they are asymptomatic, should be treated to eliminate the organism and prevent further transmission (Wright et al., 2003; Gonzales et al., 2009). However, the treatment failure and adverse drug reactions have been reported (Gardner and Hill, 2001; Harris et al., 2001; Wright et al., 2003), which frequently leading to rejection of drug (Upcroft and Upcroft, 2001; Busatti et al., 2009; Rossignol, 2010).

1.2.3.1 Amoebiasis treatment

Nitroimidazole derivatives, particularly metronidazole, are the choice of drug for amoebic colitis and amoebic liver abscess treatment. Intestinal infection is cured with metronidazole (750-800 mg) three times daily for 5-10 days or 2 g daily tinidazole for 3 days. In case of liver abscess, the patients are treated with metronidazole (750-800 mg) three times daily for 10 days and should be followed with a luminal amoebicide such as paromomycin or diloxanide furoate (Farthing, 2006). Several other drugs are also available for amoebiasis treatment such as diloxanide furoate (500 mg) three times daily for 10 days (Haque, 2003), paromomycin (25-35 mg/kg/day for 7 days) and ioquinol (650 mg) three times daily for 20 days. Consuming metronidazole and paromomycin could not give at the same time, since, commonly side effect of paromomycin is diarrhea that may make it difficult to evaluate the patient's response to treatment (Blessmann and Tannich, 2002). In amoebic liver abscess case, it might be necessary to aspiration of the abscess if the patients have no clinical response to drug therapy within 5-7 days or those who have a high risk abscess rupture that defined by a diameter of more than 5 cm diameter cavity. Furthermore, the presence of lesions in the left lobe should be considered to drain the abscess (Haque, 2003).

1.2.3.2 Giardiasis treatment

Metronidazole (2 g) is given as a single dose on three successive days was reported to cure 60-100% of gidiasis cases (Farthing, 2006). Other nitroimidazole derivatives, ornidazole and secnidazole given as a 2 g in single dose in adults and 30 mg/kg in children are also effective (Gardner and Hill, 2001). However, a single oral dose of tinidazole was approved by the Food and Drug Administration for the drug of choice to treat giardiasis in the US because of its efficacy about 90% (Watkins and Eckmann, 2014). Several types of antimicrobial chemotherapies are usable for the treatment of giardiasis for instance, Nitroimidazoles (metronidazole, tinidazole, ornidazole, secnidazole), Nitrothiazoles (nitazoxanide), Nitrofurans (furazolidone),

Acridins (quinacrine) and Benzimidazoles (albendazole, mebendazole) (Watkins and Eckmann, 2014).

Nitrothiazoles, nitazoxanide has broad-spectrum activity against intestinal parasites that clinical trials in children and adults are given 100-200 mg twice daily (efficacy 85%) (Ortiz et al., 2001).

Furazolidone is a nitrofuran compound that use for therapy of giardiasis in children, since it has few adverse effects but it has a lower efficacy than the nitroimidazole derivative. Furazolidone that has efficacy 80-96% is administered 2 mg/kg/day for 10 days in children while, adults are given 100 mg/day for 7-10 days (Gardner and Hill, 2001).

Quinacrine is an acridine derivative with 90% excellent efficacy against giardiasis. The dosage in adults is usually 100 mg, three times a day for 5-7 days while 2 mg/kg three times a day for 7 days in children (Gardner and Hill, 2001).

Benzimidazoles, albendazole, the dosage in adults are commonly 400 mg/day for 5 days and in case of mebendazole 200 to 400 mg/day for 5 to 10 days. The effectiveness of albendazole has varies markedly (25-90%) depending on the dosing regimen (Watkins and Eckmann, 2014).

1.2.4 Mechanisms of action of metronidazole against parasite interaction

In the late 1950s, metronidazole [1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole] was discovered and determined to be therapeutic against *Entamoeba histlolytica* and *Trichomonas vaginalis* (Durel et al., 1960) following it could be used to treat giardiasis that it is reported by Darbon et al. (1962). Metronidazole has nitroimidazole structure (Figure 5a) that utilizes the anaerobic metabolic pathways present in *E. histolytica* and *G. intestinalis* because its lack mitochondria.

According to a widely referred theory, metronidazole is active form when it is reduced by pyruvate: ferredoxin oxidoreductase (PFOR) and related drug with nitro groups that is toxic compound (Harris et al., 2001) as show in Figure 5b. Although, the active form of metronidazole is prevented by oxygen but it is achieved when the drug is only reduced in an anaerobic microenvironment (Lloyd and

Pedersen, 1985). Metronidazole exhibits two kind of mode of action: either it cause DNA damage or modified genes involved in the completion of mitosis.

In *G. intestinalis*, the mechanism of action of metronidazole is as described above, destroyed by leading to DNA fragmentation (Harris et al., 2001). While, *E. histolytica* and *T. vaginalis* metronidazole bind to the sulfhydyl group that involved in the thioredoxin-mediated redox network and disrupted the redox equilibrium by inhibiting thioredoxin reductase and depleting intracellular thiol pools (Leitsch et al., 2007; 2009). Moreover, several references have been reported the mechanism of metronidazole: induced apoptosis in *Blastocystis hominis* (Nasirudeen et al., 2004) and *G. intestinalis*, detached trophozoite of *G. intestinalis*, arrested the growth in S-phase by inhibiting the DNA segregation and cell division (Sandhu et al., 2004), and morphological alteration in *G. intestinalis* (Tian et al., 2006; Campanati and Monteiro-Leal, 2002). However, Oxberry et al. (1994) has reported that metronidazole caused loss of material within the cytoplasm including some glycogen and some vesicle but the nucleus was not affected in *T. vaginalis*.
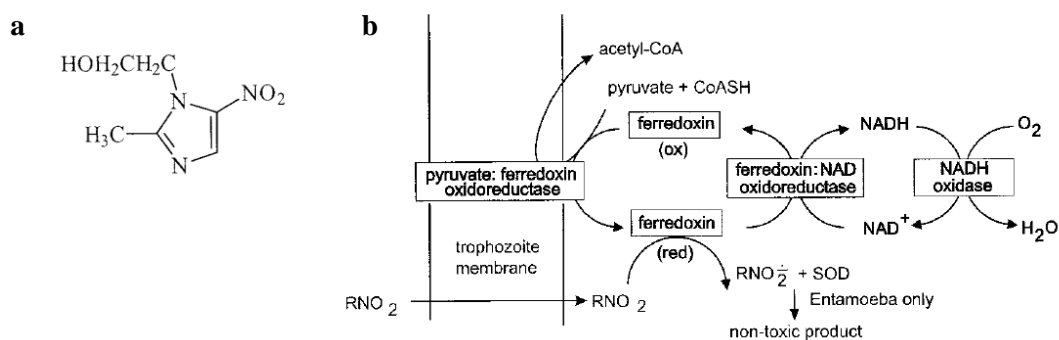
Figure 5 Structure of metronidazole (a) and anaerobic protozoa metabolism replaces pyruvate dehydrogenase (b). Nitroimidazoles (R-NO2) are activated by the parasite via a reduction to an anion radical. Electrons are regenerated by oxidative decarboxylation of PFOR, which are transferred to ferredoxin (ox). NAD received electron that catalyzed by putative ferredoxin: NAD oxidoreductase, regenerating NADH and oxidized ferredoxin. Acetyl-CoA is generated from pyruvate and reduced CoA (CoASH) in the ferredoxin reduction process. Normally NAD oxidoreductases carry out redox cycling with water being the final product (Upcroft and Upcroft, 2001).

1.2.5 The mode of drug action against parasitic protozoa

1.2.5.1 Programmed cell death

Programmed cell death (PCD) is a normal physiologic process to suicide during development and aging cell populations for removal of unwanted cells in multicellular organisms. Types of PCD known are apoptosis (type I PCD) and autophagy (type II PCD), which PCD can be initiated by various factors both external and internal signals (Mcllwain et al., 2013). Some studies have also been observed in the process of PCD in unicellular eukaryotes including *Dictyostelium* (Cornillon et al.,

1994), *Blastocystis* (Tan and Nasirudeen, 2005), *E. histolytica* (Ramos et al., 2007; Villalba et al., 2007), *G. intestinalis* (Bagchi et al., 2012), *Plasmodium* and *T. vaginalis* (Chose et al., 2003; Bruchhaus et al., 2007). The discovery of novel parasite control strategies in unicellular protozoan parasites might be investigated from PCD pathways (Elmore, 2007; Deponte, 2008).

1.2.5.1.1 Apoptosis detection

Apoptosis, or PCD, involves characteristic changes in cell morphology including cell shrinkage, DNA fragmentation, plasma membrane blebbing and phosphatidylserine exposure etc. that is caused by biochemical events leading to cell death. Apoptosis has been divided into caspase-dependent and caspase-independent pathway. The caspase-dependent pathway involves either activation of death cell receptors or the release of cytochrom c from the mitochondria. In the caspase-independent pathway involves the release of apoptosis-inducing factor and the induction of mitochondrial outer membrane permeabilization, which is controlled by the Bcl-2 family of proteins (Broker et al., 2005; Kroemer and Martin, 2005; Bagchi et al., 2012). Several methods for detections of apoptosis in cells, such as morphological changes, analysis of DNA degradation, DNA end labeling techniques or TUNEL assay, flow cytometric analysis, and electron microscopy (Sgonc and Wick, 1994; Kyrylkova et al., 2012; Martinez et al., 2010). PCD is now evident that also occurs in single-celled organisms including protists (Deponte, 2008).

The ultrastructure of *E. histolytica* determined by electron microscopy revealed that many characteristic organelles lack mitochondria, Golgi apparatus, rough endoplasmic reticulum and microtubular cytoskeleton (Ludvik and Shipstone, 1970), however, *G. intestinalis* shares a few similar characteristics with other eukaryotic cells, such as a cytoskeleton, a nucleus with nuclear envelope, and an endomembrane system (Elmendorf et al., 2003; Goldberg et al., 2008). Mitosome is a relic organelle in *E. histolytica* and *G. intestinalis* that presents some mitochondrial characteristics (Tovar et al., 2003; Leon-Avila and Tovar, 2004). In view of the fact

that a key organelle during apoptotic events is mitochondria, a cell death process of cells lacking true mitochondria could be interested.

The indicator of apoptosis in the eukaryotic cells is the expression of phosphatidylserine (PS), translocated from the inner leaflet of the plasma membrane in the outer plasma membrane which could bind to Fluorescein isothiocyanate (FITC-tagged annexin) (Shoieb et al., 2003), can be detected by annexin V assay. Annexin V is a calcium-dependent phospholipid-binding protein, which bind to PS at the external surface of the cell with high affinity for apoptosis detection. In the cell membrane of living cells, phospholipids are asymmetrically distributed between the inner and outer leaflets of the membrane. PS is located on the inner surface of membrane while phosphatidylcholine and sphingomyelin are revealed on the external leaflet of the lipid bilayer (Fadeel and Xue, 2009). During apoptosis in early stage, PS translocates to outer surface membrane, which display only Annexin V staining but the plasma membrane excludes viability dyes such as propidium iodide (PI). Cell membrane integrity loss when during late-stage apoptosis allows Annexin V binding to PS and cell uptake of PI. The characteristic green ring formation of fluorescence signal can be observed under fluorescent microscope or measured with flow cytometer. PI is a nucleic acid-specific, red-fluorescent dye that binds to DNA. PI is membrane impermeable and generally excluded from viable cells that is commonly used for discriminating between apoptotic and necrotic cells based on differences in membrane permeability (Vermes et al., 2000). Results are indicated as cells that are viable are both Annexin V-FITC and PI negative. While cells that are in early apoptosis are Annexin V-FITC positive and PI negative, and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive as shown in Figure 6.
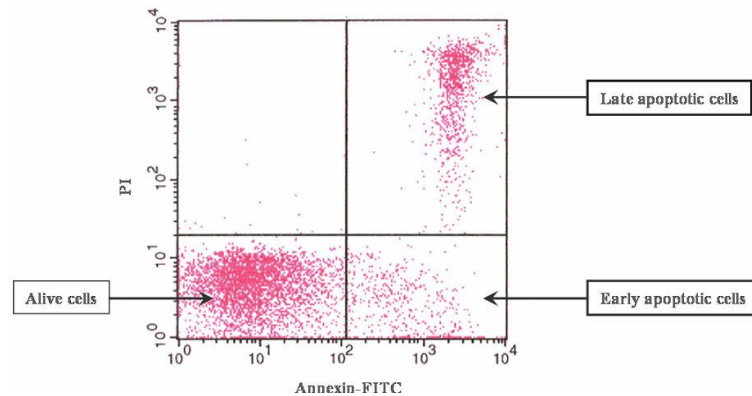
Figure 6 Three populations were detected after the Annexin V/PI staining

(Source: http://www.ro-journal.com/content/4/1/58/figure/F1)

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is acknowledged as an established method for the rapid identification and quantification of DNA fragmentation in apoptotic cells. The TUNEL assay is based on the labeled polynucleotide molecule (dUTP) to the exposed 3′-OH ends of DNA, catalyzed by exogenous terminal deoxynucleotidyltransferase. TUNEL is considered to be a sensitive method for detecting DNA fragmentation when used in conjunction with alternative techniques (Darzynkiewicz et al., 2008). Moreover, morphological changes which occur during apoptosis have identified by light and electron microscopy. The cells are smaller in size, cell shrinkage are visible during early apoptosis, alteration in cell volume, vacuolization, condensation of nuclear chromatin present in apoptotic cells and the cytoplasm is dense (Saraste and Pulkki, 2000; Elmore, 2007; Villalba et al., 2007).

1.2.6 Cell cycle analysis

E. histolytica has different cell cycle phases display the G1, S, G2/M phase unlike other eukaryotes where different phases of the cell cycle are represented as discrete fluorescent peaks representing 1n-2n genome contents (Lohia, 2003). In *G. intestinalis* during vegetative growth (trophozoite), the nuclear and cellular genome ploidy, shows each nucleus cycle between a diploid (2N) and tetraploid (4N) genome. The trophozoite multiplication is blocked in encystation process and the cell cycle in the G1-phase decreases, while G2/M population increases at the same time. (Bernander et al., 2001; Svard et al., 2002).

The cell cycle plays an important role for division and growth of parasite. Cell cycle progression is typically divided into phases G1, S, G2 and M. DNA replication occurs exclusively during S phase, such that G2-phase cells have twice the cellular DNA content compared to the G1 cells (Figure 7a). After completion of mitosis (M) the cell divides generating two daughter cells (G1) each having half the DNA content of the G2 cell. G1, S and G2/M cells can therefore be distinguished based on their DNA content that shown in Figure 7b. When DNA content is measured in a large population of cells and the data plotted in a DNA content frequency histogram, G1 and G2/M phase cells create peaks at DNA index (DI) = 1.0 and 2.0, respectively. S-phase cells are distributed in between the peaks as shown in Figure 7c. The effect of pure compound on cell cycle of protozoa will be performed using the method described by Ghosh et al. (2009).
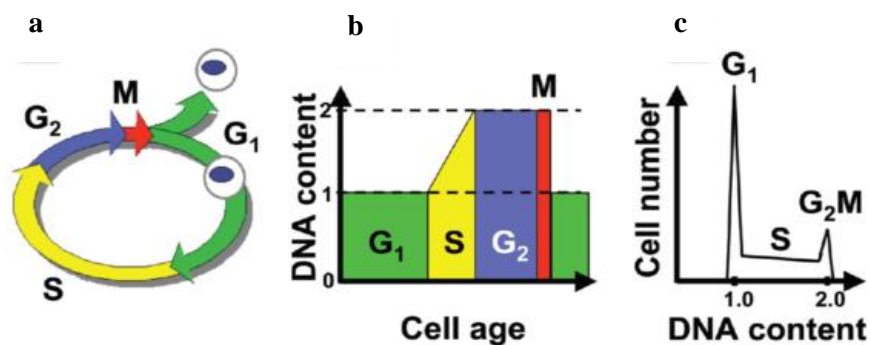
Figure 7 Phase of the cell cycle

(Source: http://www.compucyte.com/DNA_content.htm)

Interpreting cell cycle is by intercalating dye which binds to DNA and then analyzing the fluorescence properties of each cell in the population. Quiescent and G1 cells have one copy of DNA and therefore have 1X fluorescence intensity. As, the cells in S phase are synthesizing DNA, they will have fluorescence values between the 1X and 2X populations and cells in G2/M phase of the cell cycle will have two copies of DNA and accordingly will have 2X intensity (Crissman and Steinkamp, 1973; Krishan, 1975; Vielh et al., 1991) as shown in Figure 8.
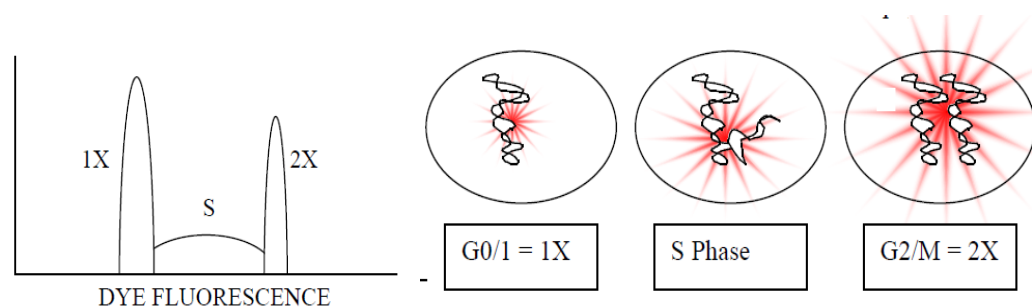
Figure 8 Diagram of DNA in different phase cells were detected after PI staining. (Source: modified from Vielh et al. (1991))

1.2.7 Adhesion capacity of protozoa

The adherence of protozoa is one of the factors that influence the pathogenesis of protozoa infection. The protein surface of *E. histolytica* is regarded to be of prime importance for host parasite interactions. The Gal/GalNAc lectin plays a central role in adhesion of the trophozoite to intestinal epithelium or in mucins, and it has been shown to be a major virulence factor (Tavares et al., 2000; Biller et al., 2014). Lectin (260-kDa) is a glycoprotein composed of a light subunit (Lgl; 31 or 35 kDa) and a heavy subunit (Hgl; 170 kDa) bound by disulfide bonds (Petri et al., 1989; Tavares et al., 2000). *In vitro*, *Giardia* trophozoites have been found to adhere to abundance of surface, including polystyrene, glass and many mammalian cell lines (Katelaris et al., 1995; Sousa et al., 2001) while *in vivo*, *Giardia* adhere to microvillus brush border of host epithelial cells. The ventral disk is a role during attachment on the surface which is considered a specific attachment organelle, made up of coiled

microtubules containing tubulin, and cross bridges that connect adjacent microtubules (Adam, 2001; Elmendorf et al., 2003; Lourenco et al., 2012); hence, the ventral disc may constitute a promising target for drug therapy.

1.2.8 Medicinal plants

The medicinal plants have been the unique source of medicines for the treatment of several diseases in different parts of the world (Hamilton, 2004; Hiremath and Taranath, 2010). Pharmaceutical research in natural products represents a major strategy for discovering and developing new drugs. The examples of the plant extracts which have been proven to have anti-amoebic or anti-girdial activity *in vitro* are shown in table 1.

Table 1 Anti-protozoal activity of plant extracts (IC_{50} and MIC) against *in vitro* growth of *Entamoeba histolytic* and *Giardia intestinalis* reported in the literatures.

plants	solvent	IC_{50} (µg/ml)		MIC		References
		E. histolytica	*G. intestinalis*	*E. histolytica*	*G. intestinalis*	
Piper longum	ethanol	ND	ND	1000 µg/ml	ND	Ghoshal et al., 1996
	chloroform fraction	ND	ND	500 µg/ml	ND	
Alpinia galanga	chloroform	55.2	37.73	ND	125 µg/ml	Sawangjaroen et al., 2005; 2006
Piper chaba	chloroform	71.4	45.47	ND	250 µg/ml	Sawangjaroen et al., 2005; 2006
Zingiber zerumbet	chloroform	196.9	69.02	ND	250 µg/ml	Sawangjaroen et al., 2005; 2006
Senna racemosa	methanol	7.15	2.10	ND	ND	Moo-Puc et al., 2007

Table 1 (cont.) Anti-protozoal activity of plant extracts (IC_{50} and MIC) against *in vitro* growth of *Entamoeba histolytic* and *Giardia intestinalis* reported in the literatures.

plants	solvent	IC_{50} (μg/ml)		MIC		References
		E. histolytica	*G. intestinalis*	*E. histolytica*	*G. intestinalis*	
Thymus vulgaris L.	a mixture of ethanol and water	ND	ND	4 mg/ml	ND	Behnia et al., 2008a
Allium sativum L.	a mixture of ethanol and water	ND	ND	60 mg/ml	ND	Behnia et al., 2008b
Ocimum basilicum L.	chloroform	68.62	53.31	ND	ND	El-Badry et al., 2010
Carica papaya	methanol	ND	ND	>62.5 μg/ml	ND	Sarker et al., 2010
Adina cordifolia	benzene	2.92	ND	ND	ND	Rani, 2011
	ethyl acetate	2.50	ND	ND	ND	

ND=No data

The anti-giardial mechanisms of compounds from plants have been demonstrated in several studies, such as inhibited trophozoite growth, effect on viability, anti-adherence of trophozoite to intestinal cell line, morphology and apoptosis-like changes. Curcumin from rhizomes of *Curcuma longa* affected *G. intestinalis* by decreasing adhesion capacity, direct cytotoxic damage and induction of a process leading to DNA fragmentation in a so-called apoptotic event (Perez-Arriaga et al., 2006). The extracts from *Mentha* x *piperita* L. or peppermint affected the growth, morphology and adhesion of *G. intestinalis* trophozoites by several alterations on its plasma membrane surface (Vidal et al., 2007). Electron microscopic studies of phenol-rich essential oils from *Thymbra capitata*, *Origanum virens*, *Thymus zygis* subsp. *sylvestris*, and *Lippia graveolens* against *G. intestinalis* indicated deformation in trophozoite appearance such as the presence of membrane blebs, electrodense precipitates in the cytoplasm and nuclei, the internalization of flagella and ventral disc (Machado et al., 2010).

The activities of the medicinal plants extract against *E. histolytica* are also extensively evaluated. Extracts from *Morinda morindoides* (Cimanga et al., 2006), Mexican traditional medicine plants (Calzada et al., 2006), seeds of *Carica papaya* (Sarker et al., 2010) and root of *Punica granatum* (Segura et al., 1990) are some example of the medicinal plants that exhibited anti-*E. histolytica* activity. However, their anti-amoeba mechanisms were rarely elucidated. Extracts and fractions from *Codiaeum variegatum* revealed differentiation, proliferation, cell growth arrest and apoptosis in *E. histolytica* (Njoya et al., 2014). Flavan-3-ol, (-)-epicatechin from *Rubus coriifolius* and *Geranium mexicanum* effected on morphological changes, induced nuclear and cytoplasmic changes and undergoing PCD in *E. histolytica* (Soto et al., 2010).

1.3   Objectives of this research

1.3.1   To screen for anti-amoebic and anti-giardial activity of the Thai medicinal plants traditionally used to treat diarrhea and parasitic infections.

1.3.2   To isolate pure compounds from promising the medicinal plant extracts and to evaluate their anti-amoebic and anti-giardial activity.

1.3.3   To study the mode of action of the promising compound with morphology, apoptotic-like changes, cell attachment and cell cycle against *E. histolytica* and *G. intestinalis*.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Methods

2.1.1 Plant materials

A total of 21 Thai medicinal plants (Table 2) which were claimed to act as agents that cured diarrhoea or parasitic infection, were evaluated for their anti-intestinal protozoan activities. These plant extracts were kindly provided by Prof. Dr. Supayang Voravuthikunchai and Assoc. Prof. Dr. Souwalak Phongpaichit, Department of Microbiology and Prof. Dr. Vatcharin Rukachaisirikul, Department of Chemistry, Prince of Songkla University. The plants were dried and extracted with acetone, ethanol or methanol as appropriate. The solvent was evaporated under reduced pressure. Each dried plant extract was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 100 mg/ml for crude extract and 10 mg/ml for pure compound, and stored at -20°C until used. In all experiments, each crude extract or pure compound was further diluted with complete medium to a require concentration. The maximum concentration of DMSO in the test did not exceed 1%, and this concentration had no effect on the *in vitro* growth of *E. histolytica* or *G. intestinalis*.

2.1.2 Pure compounds from *Coptosapelta flavescens*

The acetone extract from *C. flavescens* was the most active against *E. histolytica* and *G. intestinalis* with MIC of 125 and 15.63 µg/ml, respectively. Two anthraquinones and one napthoquinone isolated from the crude acetone extract of *C. flavescens* were then obtained from Assist. Prof. Dr. Wipapan Kongyen (Kongyen et al., 2014) for further studied.

2.1.3   Parasite cultures

        E. histolytica strain HM1: IMSS purchased from the American Type Culture Collection (ATCC®30459™) and a Thai strain of *G. intestinalis*, originally described by Siripanth et al. (1995) were used in all experiments. They were cultured axenically in screw capped tubes on YI medium (Appendix A) supplemented with 10% heat inactivated bovine serum (Gibco) at 37^oC (Diamond et al., 1995). The cultured tube was examined by inverted microscopy to monitor parasites density, and subculture was performed when they reach 80-90% confluent (approximate 48 h) by transferring 1 ml of the medium including trophozoite into a new tube and 9 ml of fresh medium was then added.

        For each assays, trophozoites were grown for 48 h and harvested by chilling the tube on ice for 15-30 min to detach the monolayer and then centrifuged at $900 \times g$ for 5 min. The supernatant was discarded, and trophozoites were resuspended in fresh medium. The numbers of viable trophozoites were calculated using a haemocytometer after stained with 0.4% (w/v) trypan blue for 5 min. The criteria for viability were motility and dye exclusion. The trophozoites were adjusted to 2×10^5 cells/ml in all experiments except for adhesion assay, the concentration of 5×10^5 cells/ml were used.

2.1.4   Screening of plant extracts and pure compounds for anti-protozoal activity

        The anti-protozoal activity was performed according to standard methods as described elsewhere (Sawangjaroen et al., 2005; 2006). Briefly, *E. histolytica* and *G. intestinalis* trophozoites (2×10^5 cells/ml) were incubated in 96-well tissue culture plates (200 µl/well) in the presence of serial two-fold dilutions of each compound that ranged from 7.81 to 500 µg/ml for crude extract and 1.25 to 80 µg/ml for pure compound. Each test also included metronidazole as the standard drug, at final concentrations ranged from 1.25 to 10 µg/ml and an untreated control (with and without 1% DMSO). Each concentration was tested in duplicate, and at least two experiments were performed on separate occasions. Trophozoites were incubated for

24 h at 37°C under anaerobic conditions (Anaerocult®A mini, Merck). After incubation, the appearance and numbers of trophozoites were scored from 1 to 4 according to the method of Upcroft and Upcroft (2001), using an inverted microscope. Score 1 showing the most inhibition of growth (<20% trophozoites cover well surface and >90% rounded up when compared to control well). Score 4 is a confluent well showing no inhibition (comparable to control well). Their minimal inhibitory concentrations (MIC) were presented as the lowest extract/drug concentration at which all wells of the same drug concentration were scored 1.

2.1.5   Time killing assay

1-hydroxy-2-hydroxymethylanthraquinone (CFQ) isolated from *C. flavescens* was further tested for its anti-protozoal activity against growth of both *E. histolytica* and *G. intestinalis* with MIC of 20 and 2.5 µg/ml, respectively. The activities of CFQ on the viability of *E. histolytica* and *G. intestinalis* at different time intervals were then compared with a standard drug, metronidazole. *E. histolytica* and *G. intestinalis* trophozoites, at a density of 2×10^5 cells/ml, were incubated at 37°C under anaerobic conditions in 96-well tissue culture plates (200 µl/well) in the absence or in the presence of metronidazole or CFQ at ½ MIC, MIC and 2 MIC concentrations for 6, 12, 24 and 48 h. After incubation, the plate was chilled for 20 min in an ice bath to detach trophozoites and their viable number was counted in a hemocytometer using trypan blue (0.4%) exclusion assay. The average number of viable trophozoite and percentage against control from each time interval were calculated. Each concentration was tested in duplicate, and at least two experiments were performed on separate occasions.

2.1.6   Determination of 50% inhibitory concentration (IC$_{50}$) and 90% inhibitory concentration (IC$_{90}$)

The IC$_{50}$ and IC$_{90}$ activities of metronidazole and CFQ were performed according to standard methods as described by Sawangjaroen et al. (2005; 2006). Briefly, trophozoites (2×10^5 cells/ml) were incubated in 96-well tissue culture plates (200 µl/well) in the presence of two-fold serial dilutions of CFQ that ranged from 1.56 to 100 µg/ml (*E. histolytica*) and 0.0976 to 6.25 µg/ml (*G. intestinalis*). Each test also included metronidazole as the standard drug, at final concentrations that ranged from 0.0781 to 10 µg/ml and an untreated control. Trophozoites were incubated for 24 h at 37°C under anaerobic conditions. After incubation, the numbers of trophozoites were determined by haemocytometer (0.4% trypan blue exclusion), counting under a light microscope. The results were calculated as the percentage of growth inhibition when compared with the controls grown without drug. The plot of the probit value against log of the compound concentration was made. The best straight line was determined by regression analysis and the concentrations that caused 50% inhibition (IC$_{50}$) and 90% inhibition (IC$_{90}$) were calculated (Appendix C). Each concentration was tested in duplicate and at least two experiments were performed on separate occasions.

2.1.7   Cytotoxicity assay

Cytotoxicity of CFQ was evaluated using African green monkey kidney as described by Hunt et al. (1999). Briefly, 45 µl the cell line (3.3×10^4 cells/ml), maintained in a minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) were added to each of plates containing 5 µl of test compound previously diluted in 0.5% DMSO, and then incubated for 4 days at 37°C in an incubator with 5% CO$_2$. Fluorescent signals were measured using a microplate reader in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm, respectively. The percentage of inhibition is calculated using the following equation, where FUT and FUC represent the

fluorescent units of cells treated with the test compound and the untreated cells, respectively:

$$\% \text{ inhibition} = [1\text{-}(\text{FUT} / \text{FUC})] \times 100$$

The compounds were considered cytotoxic when percentage inhibition is 50% or greater at the concentration of 50 μg/ml. The IC_{50} values were derived from dose-response curves, using 6 concentrations of 2 fold serially diluted samples, using SOFTMax Pro software (Molecular device). Ellipticine and 0.5% DMSO are used as positive and negative controls, respectively. The assay was performed in the National Center for Genetic Engineering and Biotechnology (BIOTEC).

2.1.8   Morphological study

2.1.8.1   Scanning electron microscopic (SEM) analysis

E. histolytica and *G. intestinalis* trophozoites (2×10^5 cells/ml) were exposed to metronidazole or CFQ at IC_{50} concentration in screw cap tube at 37°C for 24 h. Trophozoites were collected by cooling in ice for 15-30 min and centrifuged at 900 ×*g* for 5 min. The pellet was washed twice with 5 ml of PBS (pH 7.4), fixed with 2.5% glutaraldehyde in PBS (pH 7.4) for 1 h at room temperature. Following incubation, the pellet was washed twice with PBS (pH 7.4) and smeared on the glass slide (1×1 cm^2) and allowed dry. After drying, the slide was immersed in 1% Osmium tetroxide (OsO_4) for 1 h at 4°C, followed by rinsing thrice with distilled water. The samples were dehydrated with gradient of ethanol (50%, 70%, 80%, 90% and 100%) and dried by critical point drying (CPD) method (Polaron CPD7501). Samples were mounted on a stub gold-coated and trophozoites morphology was investigated by SEM (Quanta400, FEI, Czech Republic).

2.1.8.2   Transmission electron microscopic (TEM) analysis

After the protozoa were exposed to IC_{50} concentration of metronidazole or CFQ, the trophozoites were collected, fixed with 2.5% glutaraldehyde and post-fixed in 1% OsO_4 as described in 2.1.8.1. Cells were rinsed thrice with distilled water for 5 min, and stained with 2% uranyl acetate for 20 min at room temperature in the dark to enhance contrast. The samples were dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90% and 100%) and embedded in epoxy resin. Ultra-thin sections (approximately 60 nm) were then prepared with a microtome (MT-XT, RMC, USA) and stained with 5% uranyl acetate and lead citrate, followed by rinsing 2-3 times with distilled water. Samples were viewed with a transmission electron microscope (JEOL, Tokyo, Japan).

2.1.9   Mechanism of actions of active compound on *Giardia intestinalis* trophozoite

Following SEM and TEM analysis, we found that CFQ damaged *E. histolytica* cell surface causing trophozoites degeneration but the concentration of effective CFQ was quite high (4.59 µg/ml) and consider not suitable for further studied. Therefore, only the anti-giardial mechanism of metronidazole and CFQ on apoptosis induction, cell cycle analysis and anti-adhesion assay were then further explored.

2.1.9.1   Apoptosis detection

The number of apoptotic cells was determined by flow cytometry analysis after stained with annexin-V and PI. Apoptotic cells that undergo extensive DNA degradation during the early or late stage of apoptosis were confirmed by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL).

2.1.9.1.1 Flow cytometry for estimating apoptosis cell

The number of apoptosis *G. intestinalis* trophozoites after treated with metronidazole or CFQ was determined by flow cytometric analysis. *G. intestinalis* trophozoites (2×10^5 cells/ml), were treated with IC_{50} concentrations (0.42 μg/ml) of metronidazole or CFQ (0.42 μg/ml) in screw cap tube. The culture medium was used as control. The culture tubes were incubated at 37°C for 6, 12 and 24 h. Cells were stained with Annexin-V and PI using assay kit (AbD serotec, USA) in accordance with the manufacturer's instruction. Briefly, after exposure to metronidazole or CFQ, trophozoites were collected and washed twice with PBS (pH 7.4) and resuspended in binding buffer to adjust to a density of 2-5 $\times 10^5$ cells/ml. Five microlitter of Annexin V: FITC were added to 195 μl of the cells suspension, mixed and incubated for 10 min in the dark at room temperature, the cell was washed once with 190 μl binding buffer and 10 μl of PI and 190 μl binding buffer were added. The stained cells were examined under fluorescence microscopy before analyzed by flow cytrometer. Annexin V (FL1) and propidium iodide (FL3) fluorescence were measured in 10,000 cells per sample with a FC500 System flow cytometer (Beckman Coulter, USA) equipped with an air-cooled argon laser excited at 488 nm. Results were expressed as a percentage of cells in 4 categories namely; the cells did not stain with any dye ($FITC^-/PI^-$) indicated a normal cell, cells that stained only with annexin V-FITC ($FITC^+/PI^-$) indicated cells with early apoptosis, cells stained with both dyes ($FITC^+/PI^+$) and cells stained with only PI ($FITC^-/PI^+$) indicated late apoptotic and necrotic cells, respectively.

2.1.9.1.2 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL was performed using the TACS® 2 TdT-Fluor *in situ* apoptosis detection kit (Trevigen®) following the manufacturer's instructions for the detection of *in situ* DNA fragmentation. After the protozoa were exposed to metronidazole or CFQ, the trophozoites were collected and washed twice with PBS (pH 7.4) as described in 2.1.9.1.1. The pellet was fixed in 3.7% buffed formaldehyde for 10 min at room temperature, centrifuged at 500 ×*g* for 5 min, resuspended in 80% ethanol at 4°C for 1 h and approximately 10^5 trophozoites were spotted onto a clean glass microscope slide. Slides were dried for 20-60 min at 45°C on a slide warmer and immersed in 70% ethanol for 10 min and air dried overnight at room temperature. Slides were rehydrated in decreased series of ethanol (100%, 95% and 70%) by immersing the slide for 5 min each and immersed in PBS (pH 7.4) for 10 min at room temperature. Samples were covered with 50 µl Proteinase K (1 mg/ml) solution and incubated for 30 min at room temperature. After incubation, the slides were washed twice with deionized water for 2 min each. For positive control slide, after treated with proteinase K, untreated trophozoites were exposed to 50 µl of TACS-Nuclease™ solutions (TACS Nuclease 1:50 in the TACS Nuclease buffer) and incubated for 60 min at room temperature in a humidity chamber to generate DNA breaks in every cell. Both samples and positive control slides were immersed in TdT labeling buffer for 5 min, covered with 50 µl of labeling reaction mix and incubated at 37°C for 1 h in a humidity chamber. The slides were then immersed in TdT stop buffer for 5 min at room temperature, washed twice with deionized water for 5 min each and then incubated in TUNEL reaction (50 µl of Strep-Fluor solution) for 20 min at room temperature in the dark. Following incubation, the slides were washed twice in PBS for 2 min each. The samples were viewed with a fluorescence microscope. The cells display TUNEL-positive cell green fluorescence in the nucleus indicated early apoptotic events. In the late stage of apoptotic, cells are displayed as condensed and fragmented orange chromatin.

2.1.9.2   Cell cycle analysis

The DNA content of *G. intestinalis* after treated with IC_{50} concentration of metronidazole or CFQ was determined by a method described by Perez et al. (2001) with some modification. *G. intestinalis* trophozoite was treated with IC_{50} concentration of metonidazole or CFQ as described in 2.1.9.1.1 for 6, 12 and 24 h. The trophozoites were harvested and centrifuged; then the pellet was washed twice with PBS (pH 7.4), and fixed by adding drop by drop of 1 ml ice-cool 70% ethanol with gentle mixing and left at 4°C for 30 min. The fixative was decanted after centrifuged at 850 ×*g* for 5 min and the pellet was washed once with PBS (pH 7.4). The cells were incubated at 37°C for 30 min in the dark with RNase (Invitrogen) at 100 μg/ml and PI (Invitrogen) at 5 μg/ml. Percentage of each cell cycle phase: Sub-G1 peak which indicated apoptotic cells, G1 (the first stage, cell monitor their environment and internal signals that aim to replicate/do not replicate decision), S (DNA synthesis), and G2/M (G2/M defined as the second gap between S and mitosis as DNA damage repair, and preparation for entering mitosis (M phase)) were analyzed by flow cytometry (FC500, Beckman Counter, USA) from DNA content of 10,000 trophozoites.

2.1.9.3 Inhibition of *Giardia intestinalis* adhesion to Caco-2 cell line

The adherent protozoa are one of the factors influence the pathogenesis of protozoa infection. The effect of CFQ to inhibit adhering ability of protozoa to intestinal cell line was determined by a modified co-culture according to Katelaris et al. (1994) and Sousa et al. (2001). Colon cancer cells, Caco-2 cell line (ATCC HTB-37[TM]), kindly provided by Assoc. Prof. Dr. Teerapol Srichana, faculty of Pharmaceutical Sciences, Prince of Songkla University, were used in this study. The cells were cultured in 25 cm^2 flask in minimum essential medium (MEM, Gibco) containing 20% fetal bovine serum (Gibco), 1% v/v streptomycin (100 μg/ml, Gibco) and penicillin (100 units/ml, Gibco) at 37°C in 5% CO_2 incubator. The medium was changed every 48 h and the cultured flask was determined every day by inverted microscopy to monitor cells density. Subculture was performed when the cells reach 80-90% confluent (approximate 7 days) by removing the media and washed once with 5 ml PBS (pH 7.4) to remove excess medium and serum. The cells were detached from the bottom of the flask by adding 1 ml of 0.25% trypsin-EDTA (Gibco) and incubated at 37°C for 5 to 10 min or until cells are detached. In order to stop the reaction, 4 ml of fresh media (containing serum and antibiotics) was added to the flask and the cells were resuspended in the media by gentle pipetting over the surface of the flask. Resuspended cells (1 ml) were transferred into a new flask containing 4 ml of fresh medium and incubated under 5% CO_2 at 37°C.

For anti-adhesion assay, Caco-2 cells were grown in 24-well tissue culture plate at a density of 1×10^5 cells/well and incubated at 37°C with 5% CO_2 until confluent monolayer were developed (4-6 days; approximately 5×10^6 cells/well).

Co-culture was done at the cell ratio (parasite: Caco-2 cell) approximately 1:10. Briefly, 1 ml *G. intestinalis* trophozoite (5×10^5 cells/ml) in MEM medium was added to 24-well plate containing confluent Caco-2 cell line and incubated at 37°C in 5% CO_2 for 1 h to allow *G. intestinalis* to adhere on the Caco-2 cell line. Unattached trophozoites were discarded by gently rinsing the culture plate once with warm (37°C) MEM medium. IC_{50} concentration of metronidazole or CFQ were added in the plates and incubated at 37°C in 5% CO_2 for 6, 12 and 24 h.

Unattached trophozoites were discarded and gently rinsed twice with warm (37°C) MEM medium. Adherent trophozoites were then recovered by repeated washing with ice cold PBS (pH 7.4) over 15-30 min or until no trophozoites were seen under light microscopy. The number of attached trophozoite from each sample was counted with a heamocytometer, and the percentage of attachment was calculated as follows (modified from Cruz et al., 2003):

$$\% \text{ attachment} = \frac{\text{attached trophozoites from test well}}{\text{attached trophozoites from control well}} \times 100$$

2.1.10 Statistical analysis

The t test and ANCOVA (Analysis of Covariance) procedures were used to compare the mean values of experimental groups in time kill assay and anti-adhesion assay, respectively. A P-value of <0.05 was considered as statistically significant.

2.2 Materials and Equipments

2.2.1 Microorganisms

- *Entamoeba histolytica* HM1:IMSS (ATCC®30459™)
- *Giardia intestinalis* (Thai stain)

2.2.2 Chemicals

- Ascorbic acid	(Riedel)
- Bovine serum	(GIBCO)
- Calcium pantothenate	(SIGMA)
- Conc. H_2SO_4	(LAB SCAN)
- Crystal violet	(MERCK)
- d-Biotin	(SIGMA)
- Dehydrated bovine bile	(SIGMA)
- Dimethy sulfoxide (DMSO)	(MERCK)
- DL-6,8 thioctic acid	(SIGMA)
- Ferric ammonium citrate	(SIGMA)
- Folic acid	(SIGMA)
- Glucose	(SIGMA)
- HCl	(BDH)
- K_2HPO_4	(MERCK)
- KH_2PO_4	(SIGMA)
- L-cysteine hydrochloride	(ALDRICH)
- Methanol	(BDH)
- Metronidazole	(SIGMA)
- NaCl	(BDH)
- Na_2HPO_4	(MERCK)
- NaOH	(MERCK)
- Phosphate buffer saline	(SIGMA)
- Pyridoxal hydrochloride	(SIGMA)

- Propidium iodide   (Invitrogen)
- Riboflavin   (SIGMA)
- RNase   (Invitrogen)
- Thianium hydrochloride   (SIGMA-ALDRICH)
- Trypsin EDTA   (GIBCO)
- Versene   (GIBCO)
- Tween 80   (SIGMA)
- Vitamine B12   (SIGMA)
- Yeast extract   (MERCK)

2.2.3 Media

- Bovine serum   (GIBCO)
- Fetal bovine serum   (GIBCO)
- Minimal Essential Medium (MEM)   (GIBCO)
- Vitamin mix 18   (Appendix A)
- YI-S medium   (Appendix A)

2.2.4 Antibiotics

- Metronidazole   (SIGMA)
- Penicilin/streptomycin   (GIBCO)

2.2.5 Kits

- Annexin V:FITC assay kit   (AbD serotec)
- TACS$^{®}$2 TdT Fluor In Situ   (TREVIGEN)
  Apoptosis Detection kit

2.2.6 Instruments

- Anaerocult® A mini (MERCK)
- Autoclave (Tomy)
- Biosafety cabinet (Microflow)
- Centrifuge (HERMLE Z200A)
- Counting chambers (Boeco)
  (Haemacytometer)
- Eppendorf tube (Axygen)
- Flow cytometer (Beckman)
- Fluorescence microscope (Olympus DP71)
- Incubator (Heraeus GmbH)
- Inverted Microscope (Olympus)
- Light Microscope (Olympus)
- Inverted Microscope (Olympus)
- Micro pipette (Eppendorf)
- Millipore filter (0.2, 0.45 µm) (Sartorius)
- Pasture pipette (Volac)
- pH meter (Backman)
- Tip 20, 200, 1000 and 5000 ml (Axygen)
- Tissue culture flask (25, 75 cm^2) (SPL)
- Water bath (Julabo)
- 24-well tissue culture plate (SPL)
- 96-well tissue culture plate (NUNC)

CHAPTER 3

RESULTS

3.1 Anti-protozoal activity of the plant extracts

The Minimal Inhibitory Concentration (MIC) of each plant extract against *in vitro* growth of *E. histolytica* and *G. intestinalis* are summarized in Table 2. The full information of scoring given to each concentration of the extract and metronidazole are shown in Appendix B. The acetone extract from *C. flavescens* showed the most potent activity against both *E. histolytica* and *G. intestinalis* with MICs of 125 and 15.63 µg/ml, respectively. The ethanol extract of *C. longa* inhibited both *E. histolytica* and *G. intestinalis* at a concentration of 250 µg/ml, whereas *E. thymifolia*, *G. mangostana*, *P. granatum*, *S. koetjape* and *T. bellerica* inhibited both the protozoa at 250-500 µg/ml. *Derris scandens* inhibited only *E. histolytica* (MIC 500 µg/ml). *Piper betle*, *P. guajava*, *R. mucronata* and *T. chebula* inhibited only *G. intestinalis* at 250-500 µg/ml. The remaining extracts showed no activity (MIC ≥500 µg/ml). The MIC value of metronidazole against both *E. histolytica* and *G. intestinalis* was 2.5 µg/ml.

Table 2 Name of the plants, their parts used and MIC values of plant crude extracts against *Entamoeba histolytica* and *Giardia intestinalis*.

Plants	Part used	Extraction solvent	MIC (µg/ml)	
			E. histolytica	*G. intestinalis*
Aegel marmelos (L.) Corr.	fruit	ethanol	>500	>500
Ardisia colorata Roxb.	wood	ethanol	>500	>500
Centella asiatica (L.) Urb.	whole plant	ethanol	>500	>500
Coptosapelta flavescens	root	methanol	>500	>500
	whole plant	acetone	125	15.63
Curcuma longa L.	rhizome	ethanol	250	250
Derris scandens Roxb. Benth.	stem	ethanol	500	>500
Euphorbia thymifolia L.	whole plant	ethanol	500	500
Garcinia mangostana L.	skin	ethanol	500	500
Holarrhena pubescens Wall. ex G. Don	bark	ethanol	>500	>500
Manilkara achras (Mill.) Fosberg	fruit	ethanol	>500	>500
Morinda citrifolia L.	fruit	ethanol	>500	>500
Peltophorum pterocarpum (DC.) Backer. ex K.Heyne.	bark	ethanol	>500	>500
Piper aurantuacum	leaf	ethanol	>500	>500
Piper betle L.	leaf	ethanol	>500	500
Piper chaba Vahl.	fruit	ethanol	>500	>500
Psidium guajava L.	leaf	ethanol	>500	500
Punica granatum L.	skin	ethanol	500	250
Rhizophora mucronata Poir	bark	ethanol	>500	500
Sandoricum koetjape (Burm. f.) Merr.	root	ethanol	500	500

Table 2 (cont.) Name of the plants, their parts used and MIC values of plant crude extracts against *Entamoeba histolytica* and *Giardia intestinalis*

Plants	Part used	Extraction solvent	MIC (µg/ml)	
			E. histolytica	*G. intestinalis*
Terminalia bellerica (Gaertn.) Roxb.	fruit	ethanol	500	500
Terminalia chebula Retz.	fruit	ethanol	>500	250
metronidazole			2.5	2.5

3.2　Chemical constituents of *Coptosapelta flavescens*

From the screening study, the acetone extract from *C. flavescens* was most active against the tested protozoa. Thus, only the extract of *C. flavescens* was subjected to further purification step. Two anthraquinones (Compound **1** and **2**) and one naphthoquinone (Compound **3**) (Figure 9) were obtained from the crude acetone extract of *C. flavescens* (Kongyen et al., 2014). Compound **1, 2** and **3** were identified as 2-amino-3-methoxycarbonyl-1,4-naphthoquinone, 1-hydroxy-2-hydroxymethylanthraquinone (CFQ) and 1-hydroxy-2-methoxycarbonylanthraquinone, respectively.

2: R_1 = OH, R_2 = CH$_2$OH, R_3 = H

3: R_1 = OH, R_2 = CO$_2$Me, R_3 = H

1
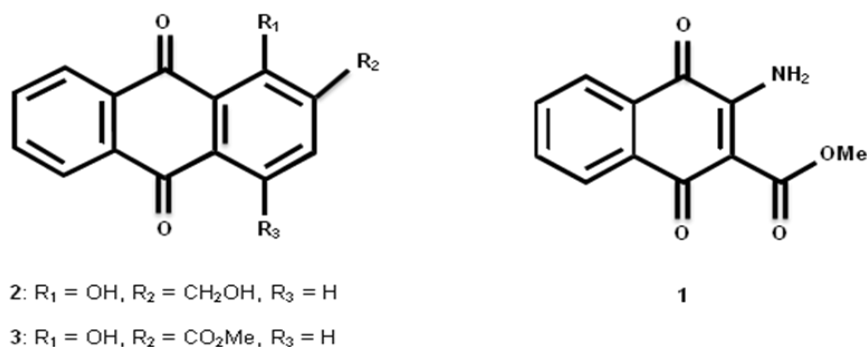
Figure 9 Chemical constituents of *Coptosapelta flavescens*

3.3 *In vitro* **anti-amoebic and anti-giardial activity of pure compounds isolated from** *Coptosapelta flavescens*

The *in vitro* effects of each pure compound isolated from *C. flavescens* against *E. histolytica* and *G. intestinalis* are shown in Table 3. The MIC of compound **2** (CFQ) against *E. histolytica* and *G. intestinalis* was 20 and 2.5 µg/ml, respectively. The morphology and approximate number of *E. histolytica* and *G. intestinalis* treated with metronidazole and CFQ at the MIC concentration for 24 h under inverted microscope are shown in Figure 10b, c and 11b, c, respectively, while the trophozoites from the control well were more than 90% confluent (Figure 10a and 11a). Compound **1** and compound **3** showed MIC values of >80 µg/ml and thereby excluded from further study. IC_{50} and IC_{90} values of metronidazole and CFQ, using probit analysis, against *E. histolytica* and *G. intestinalis* are given in Table 4. IC_{50} of metronidazole and CFQ against *G. intestinalis* are similar at 0.42 ± 0.21 µg/ml and 0.42 ± 0.05 µg/ml, while CFQ against *E. histolytica* showed less activity with IC_{50} of 4.59 ± 1.92 µg/ml.

Table 3 The minimal inhibitory concentrations (MICs) of pure compounds isolated from *Coptosapelta flavescens* against *Entamoeba histolytica* and *Giardia intestinalis in vitro*.

Compounds	MIC (µg/ml)	
	E. histolytica	*G. intestinalis*
2-amino-3-methoxycarbonyl-1,4-naphthoquinone (compound **1**)	>80	>80
1-hydroxy-2-hydroxymethylanthraquinone (compound **2, CFQ**)	20	2.5
1-hydroxy-2-methoxycarbonylanthraquinone (compound **3**)	>80	>80
Metronidazole	2.5	2.5

Table 4 The IC_{50} and IC_{90} of 1-hydroxy-2-hydroxymethylanthraquinone isolated from *Coptosapelta flavescens* against *Entamoeba histolytica* and *Giardia intestinalis in vitro*.

Compounds	Mean IC_{50} (µg/ml) ± SD		Mean IC_{90} (µg/ml) ± SD	
	E. histolytica	*G. intestinalis*	*E. histolytica*	*G. intestinalis*
CFQ	4.59 ± 1.92	0.42 ± 0.05	47.33 ± 46.81	3.05 ± 0.68
metronidazole	0.44 ± 0.16	0.42 ± 0.21	2.56 ± 1.06	9.11 ± 4.40
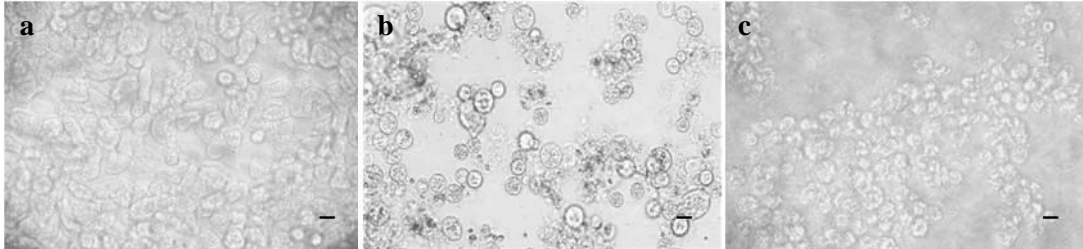
Figure 10 *Entamoeba histolytica* trophozoites after treatment with minimal inhibitory concentration of (a) untreated control, (b) metronidazole (2.5 µg/ml) and (c) 1-hydroxy-2-hydroxymethylanthraquinone (20 µg/ml) at 37°C for 24 h. Bars = 10 µm.
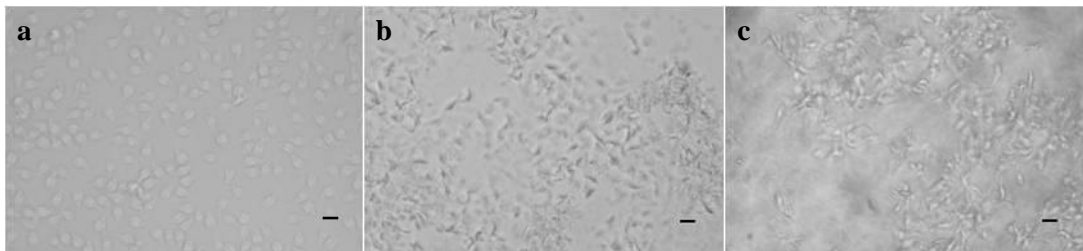
Figure 11 *Giardia intestinalis* trophozoites after treatment with minimal inhibitory concentration of (a) untreated control, (b) metronidazole (2.5 µg/ml) and (c) 1-hydroxy-2-hydroxymethylanthraquinone (2.5 µg/ml) at 37°C for 24 h. Bars = 10 µm.

3.4   Time killing assay

The percentage of viable *E. histolytica* and *G. intestinalis* after exposed to metronidazole and CFQ at ½ MIC, MIC and 2 MIC concentrations for 6, 12, 24 and 48 h when compared to the control at the same time interval, are shown in Figure 12 and 13, respectively. Metronidazole and CFQ at MIC concentration decreased number of viable *E. histolytica* and *G. intestinalis* trophozoites to <20% within 24 h. However, it is interesting to note that at 6 and 12 h the percentage of viable *G. intestinalis* exposed to CFQ significantly ($p<0.05$) lower than when exposed to metronidazole.

3.5   Cytotoxicity test

The IC_{50} of Compound **1, 2** and **3** against Vero cells (African green monkey kidney) are shown in Table 5. Their IC_{50} are <50 µg/ml indicated cytotoxic to Vero cells.

Table 5 The IC_{50}s of pure compounds isolated from *Coptosapelta flavescens* and ellipticine against Vero cells.

Compounds	IC_{50} (µg/ml)
2-amino-3-methoxycarbonyl-1,4-naphthoquinone (compound **1**)	48.93
1-hydroxy-2-hydroxymethylanthraquinone (compound **2, CFQ**)	18.42
1-hydroxy-2-methoxycarbonylanthraquinone (compound **3**)	7.09
Ellipticine	0.860
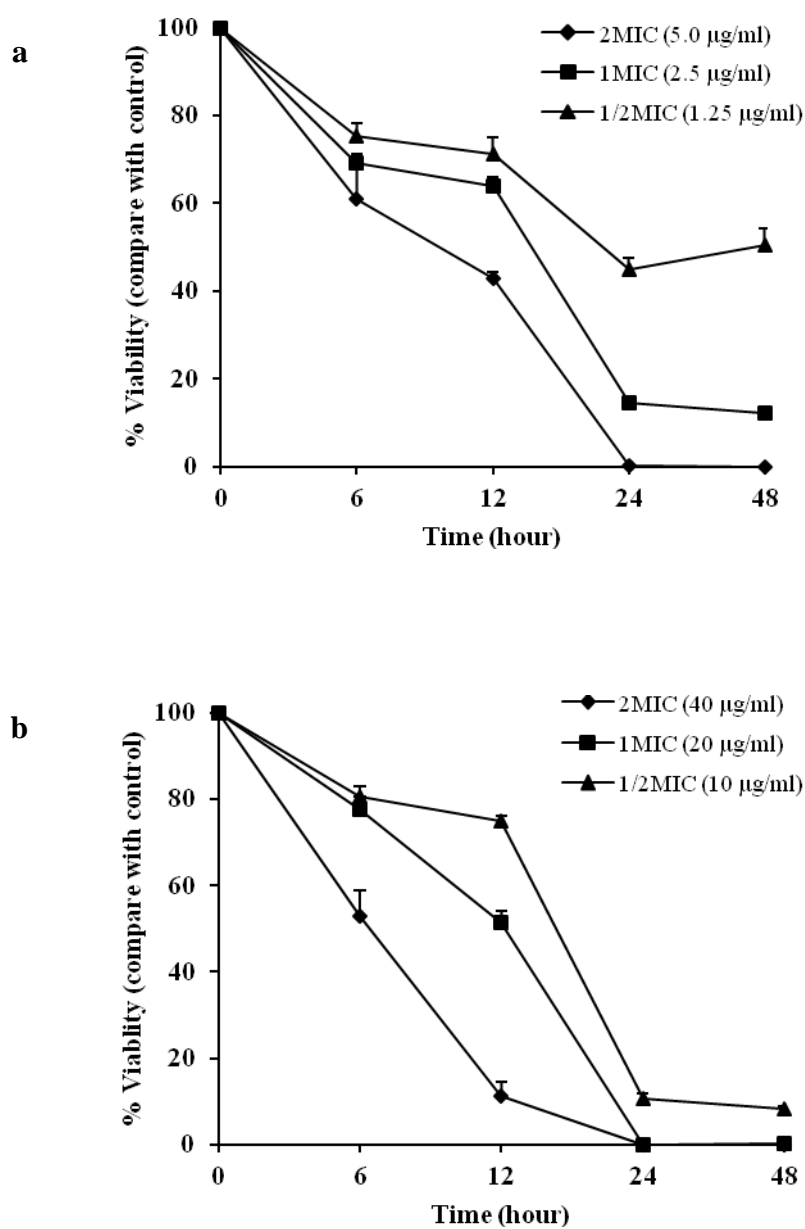
Figure 12 Percentage of viable *Entamoeba histolytica*, compared to control, after incubation with different concentrations of (a) metronidazole and (b) 1-hydroxy-2-hydroxymethylanthraquinone for 6, 12, 24 and 48 h. Values are expressed as means ± standard error of the mean.
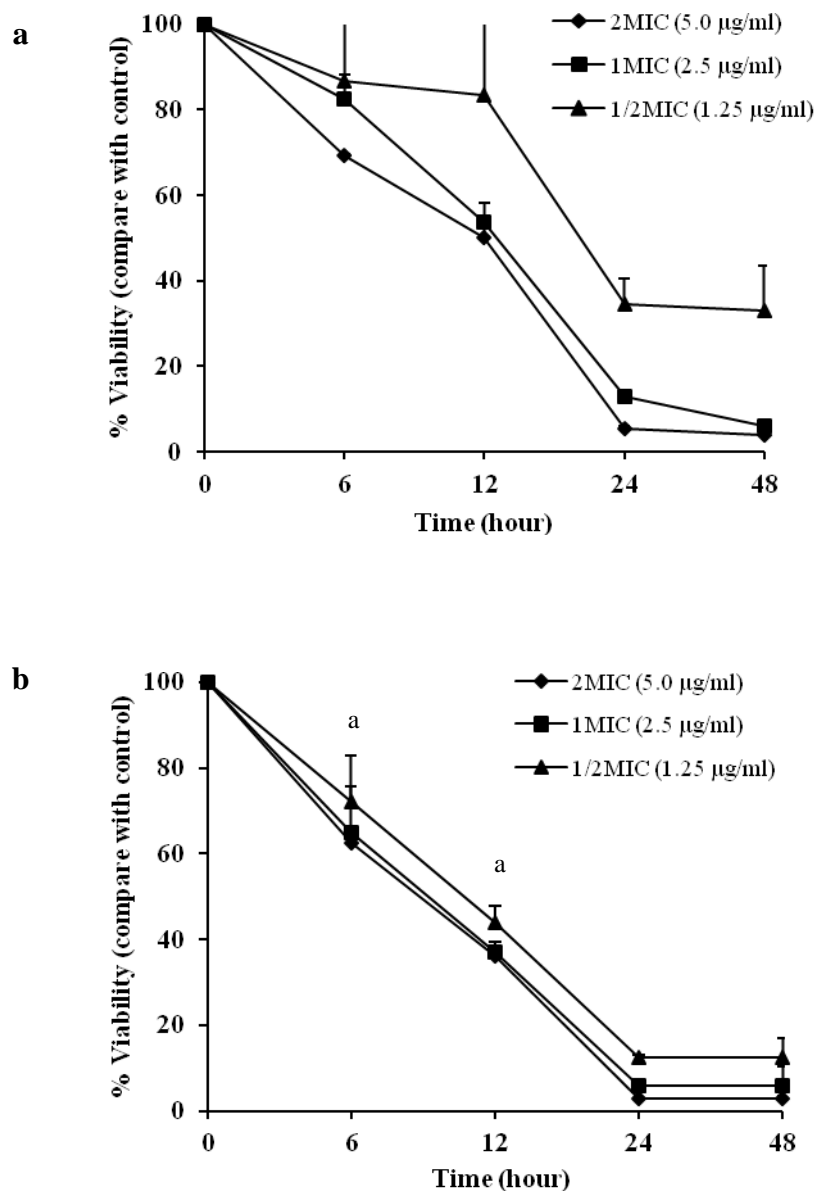
Figure 13 Percentage of viable *Giardia intestinalis*, compared to control, after incubation with different concentrations of (a) metronidazole and (b) 1-hydroxy-2-hydroxymethylanthraquinone for 6, 12, 24 and 48 h. Values are expressed as means ± standard error of the mean, [a]$p<0.05$.

3.6   Effects of 1-hydroxy-2-hydroxymethylanthraquinone on morphology of
Entamoeba histolytica **and** *Giardia intestinalis*

3.6.1 Scanning electron microscopy

Under scanning electron microscope, after 24 h incubation untreated *E.*
histolytica trophozoite showed spherical shape with smooth cell surface (Figure 14).
In contrast, after exposed to metronidazole at its IC_{50} concentration (0.44 µg/ml) for
24 h, outer membrane of most *E. histolytica* was blebbling (black arrows) with several
holes on the surface (black arrowheads) (Figure 15a and b). *E. histolytica* trophozoite
incubated with CFQ at IC_{50} concentration (4.59 µg/ml) for 24 h appears rough cell
surface, membrane bleb (black arrows) with some large hole (black arrowheads)
(Figure 16a and b).

The untreated *G. intestinalis* trophozoites (Figure 17) revealed a pear-
shaped body with a typical cytoskeleton including pairs of anterior (A), ventral (V),
posterior-lateral (PL), caudal flagella (C), lateral flange (LF), ventrolateral flange
(VF) and ventral disk (VD). Following 24 h exposures to 0.42 µg/ml metronidazole
(Figure 18), most trophozoites had an irregular shape of ventral and dorsal surface
(asterisks) as well as a bulbous structure in the flagella end and cell membrane (white
arrowheads). In the presence of 0.42 µg/ml CFQ, *G. intestinalis* trophozoites became
completely deformed; most cells presented abnormal morphology, had an irregular
shape of their ventral and dorsal surface (asterisks), VD and membrane was clearly
rupture (black arrowhead) (Figure 19a) including hole on dorsal surface (Figure 19b).
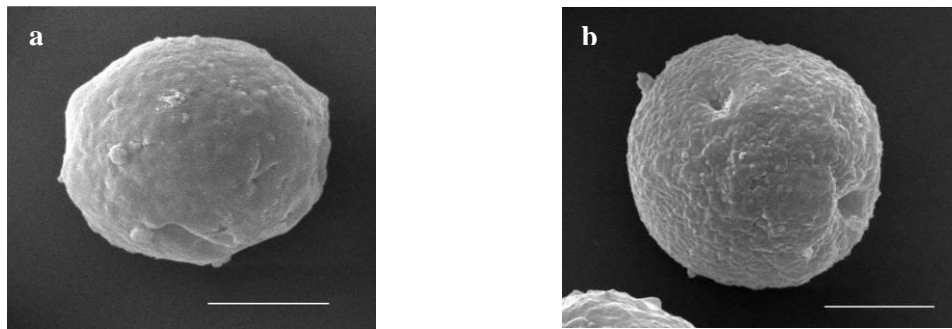
Figure 14 Scanning electron micrograph of normal *Entamoeba histolytica* trophozoite culture for 24 h in (a) complete medium and (b) complete medium containing 1% DMSO. Note the trophozoite displays spherical shape with smooth cell membrane. Bars = 5 μm.
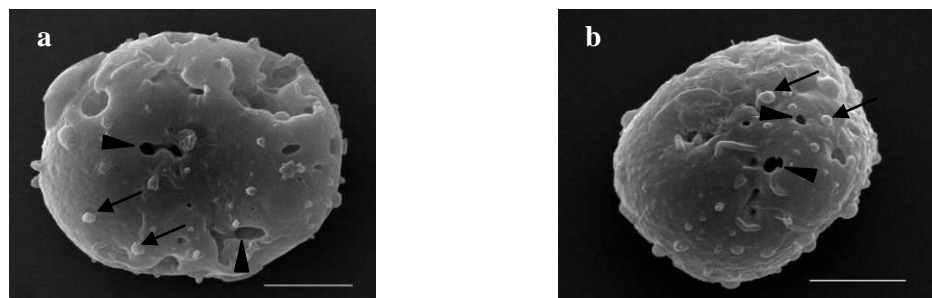
Figure 15 Scanning electron micrograph of *Entamoeba histolytica* treated with metronidazole 0.44 μg/ml for 24 h. Note the holes on their cell surface (black arrowheads) and membrane blebs (black arrows). Bars = 5 μm.
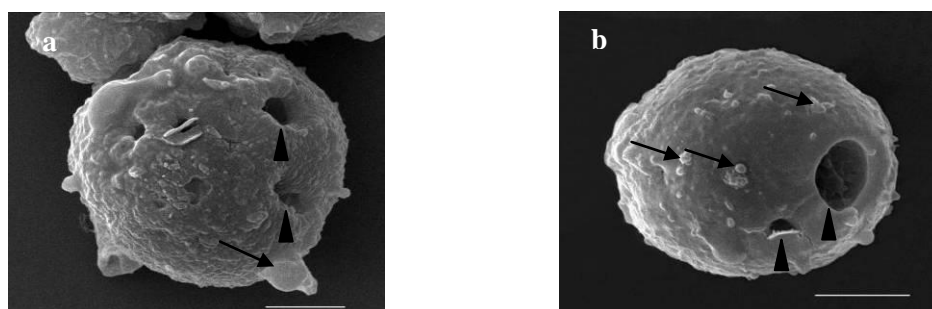
Figure 16 Scanning electron micrograph of *Entamoeba histolytica* treated with 1-hydroxy-2-hydroxymethylanthraquinone 4.59 μg/ml for 24 h. Note the holes on their cell surface (black arrowheads) and membrane blebs (black arrows). Bars = 5 μm.
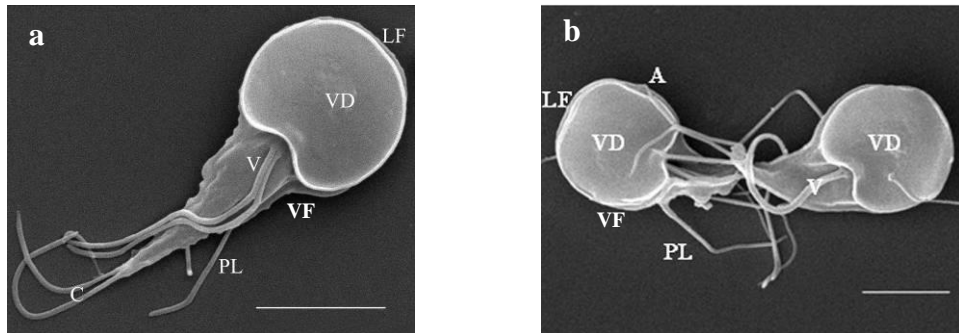
Figure 17 Scanning electron micrograph of normal *Giardia intestinalis* trophozoite culture for 24 h in (a) complete medium and (b) complete medium containing 1% DMSO. Note trophozoites display half-pear morphology, pairs of anterior flagella (A), ventral flagella (V), lateral flange (LF), ventrolateral flange (VF), posterior–lateral flagella (PL), caudal flagella (C), and one ventral disk (VD). Bars = 5 μm.
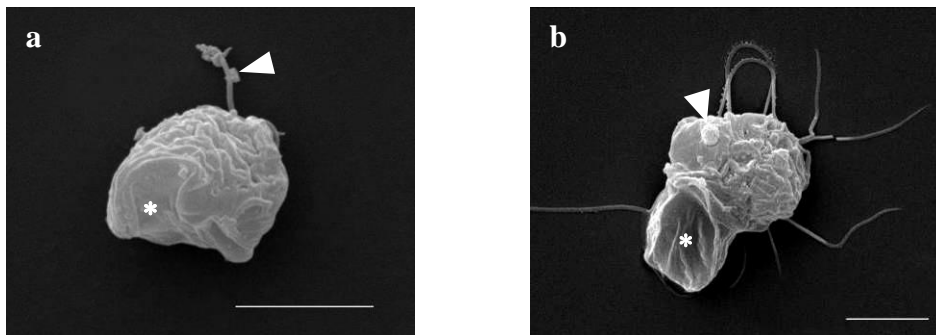
Figure 18 Scanning electron micrograph of *Giardia intestinalis* treated with metronidazole 0.42 μg/ml for 24 h. Note the round shape, irregular ventral and dorsal surface (asterisks), bulbous structure in the flagella end and cell membrane (white arrowheads). Bars = 5 μm.
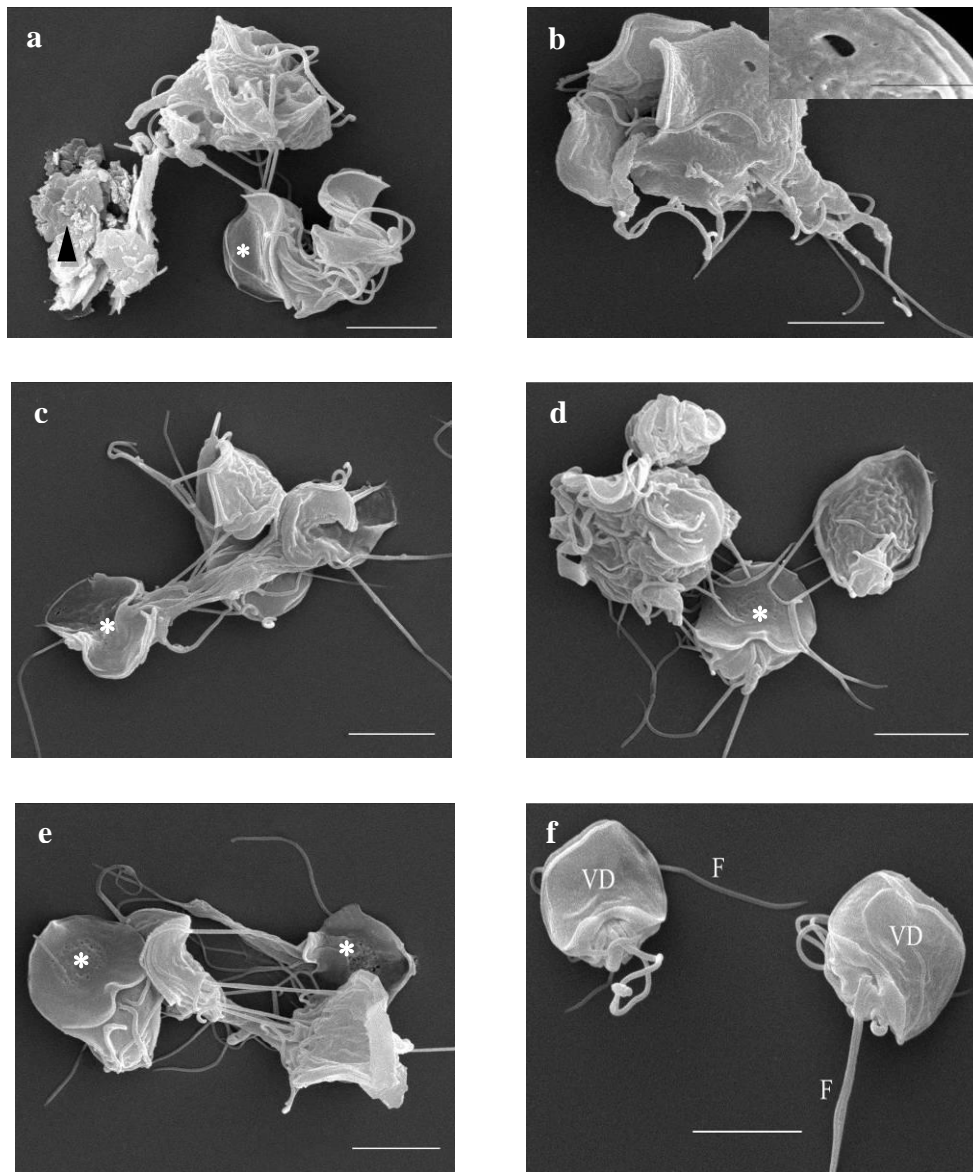
Figure 19 Scanning electron micrograph of *Giardia intestinalis* treated with 1-hydroxy-2-hydroxymethylanthraquinone 0.42 μg/ml for 24 h. Note the round shape, irregular ventral and dorsal surface (asterisks) and membrane rupture (black arrowheads). Bar = 5 μm.

3.6.2 Transmission electron microscopy

Ultrastructure of untreated *E. histolytica* trophozoites under TEM are shown in Figure 20a, b and c. The intact plasma membrane is clearly observed (open triangles) with the typical small accumulations of cytoplasmic glycogen (G), clear vacuoles (V), as well as some vacuoles that contain debris in the form of membrane fragments (open circles). Moreover, the amoeba nucleus (N) demonstrated peripherally localized chromatin. After treated with metronidazole at IC_{50} for 24 h cells still exhibited continuous plasma membrane without alterations (open triangles) but increased number of vacuole and large cytoplasmic vacuolations (asterisks) were indicated (Figure 21a and b). Nuclear condensation was also observed in some trophozoites (black arrowheads) that are shown in Figure 21c. Under CFQ treatment, the number of glycogen content (G) was greater than in control or metronidazole treatment. In *E. histolytica* trophozoite treated with CFQ for 24 h, showed irrugular plasma membrane surface (open triangles), breaking of plasma membrane as indicated by excretion of cellular material (arrows) (Figure 22a and b) and membrane rupture are clearly observed (Figure 22c). The fragmented nucleus and dense chromatin and the round nuclear bodies were more conspicuous (black arrowhead) (Figure 22a and b).

For *G. intestinalis*, under TEM normal trophozoites presented with two nuclei (N) that revealed more electron dense material, ventral disk (VD) composed of numerous microtubules, peripheral vesicle (PV) aligned at dorsal surface and axonemes (A) of six posterior flagella are seen between the two nuclei (Figure 23a and b). After being treated with 0.42 µg/ml of metronidazole for 24 h (Figure 24a, b and c) the trophozoites had membrane blebs (open triangle), disruption of their ventrolateral flange (open circle), fragmentation of VD, an increasing volume of PV (cross). The number of vacuole in the cytoplasm (asterisks) is also increased, and nucleus presented with condensation of chromatin (black arrowheads). Ultrastructure of *G. intestinalis* trophozoite treated with CFQ is shown in Figure 25a, b and c. The cells are misshapen (Figure 25a), presence of large vacuoles in the cytoplasm (asterisks), fragmentation of ventral disk (square), and presence of electrodense

precipitates in cytoplasm and nuclei (black arrowheads). These results indicated that CFQ may induce apoptosis in *G. intestinalis* trophozoite.
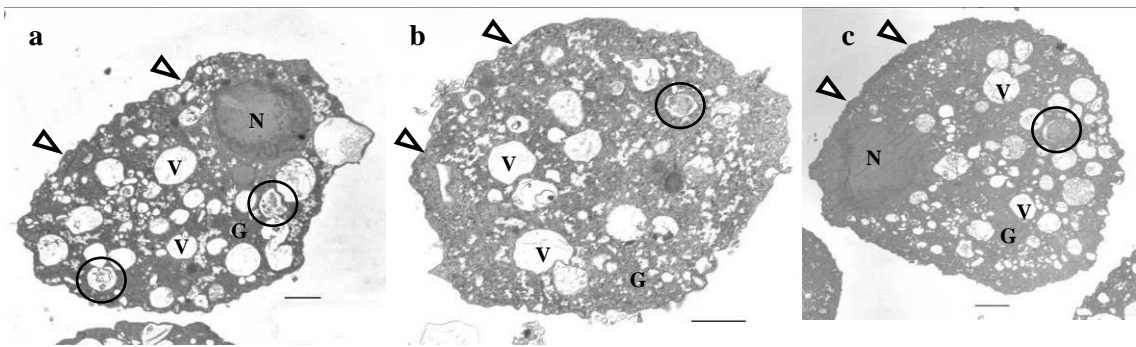
Figure 20 Transmission electron microscopic image showing normal *Entamoeba histolytica* trophozoites. Note intact plasma membrane (open triangles) and vacuoles containing debris (open circles). Bars = 2μm.

V = vacuoles, N = nucleus, G = glycoprotein
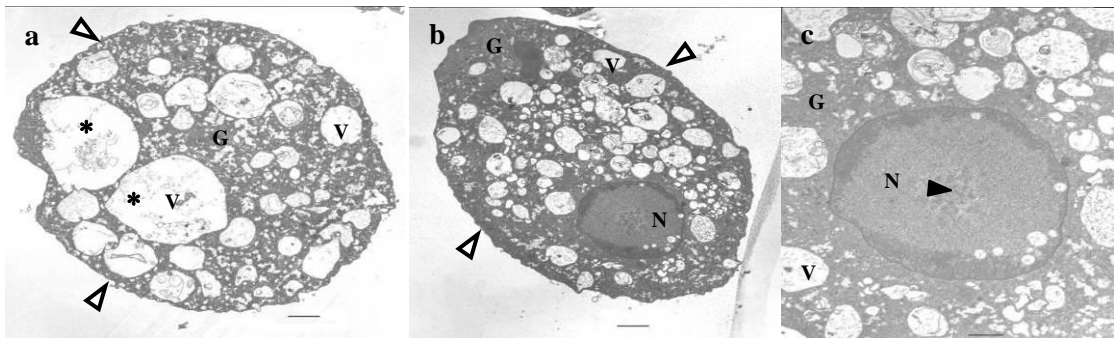
Figure 21 Transmission electron microscopic image showing *Entamoeba histolytica* treated with 0.44 µg/ml metronidazole for 24 h. Note intact plasma membrane (open triangles), large cytoplasmic vacuolations (asterisks) and dense chromatin (black arrowhead). a and b, bars = 2 µm; c, bar = 1μm.
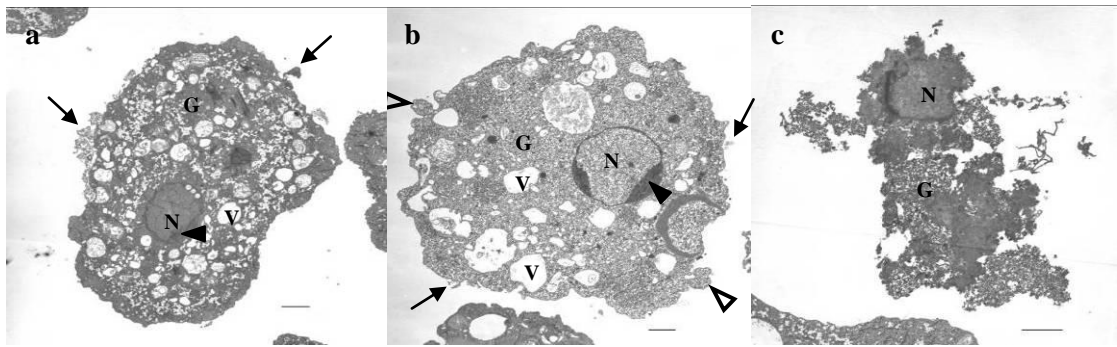
Figure 22 Transmission electron microscopic image showing *Entamoeba histolytica* trophozoite treated with 4.59 µg/ml 1-hydroxy-2-hydroxymethylanthraquinone for 24 h. Note irrugular plasma membrane surface (open triangles), breaking of plasma membrane (arrows), dense chromatin and the round nuclear bodies (black arrowhead). Bars = 2 μm.
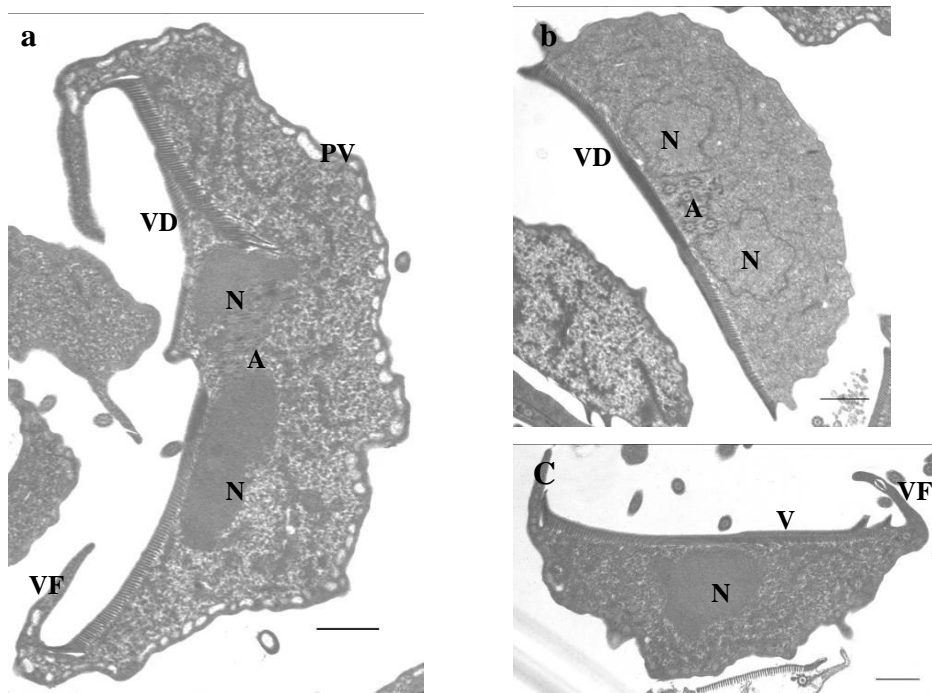
Figure 23 Transmission electron microscopic image showing untreated *Giardia intestinalis* trophozoites after 24 h of incubation. Bars = 1μm

A = axonemes, N = nucleus, PV = peripheral vesicle, VF = ventrolateral flange and VD = ventral disk
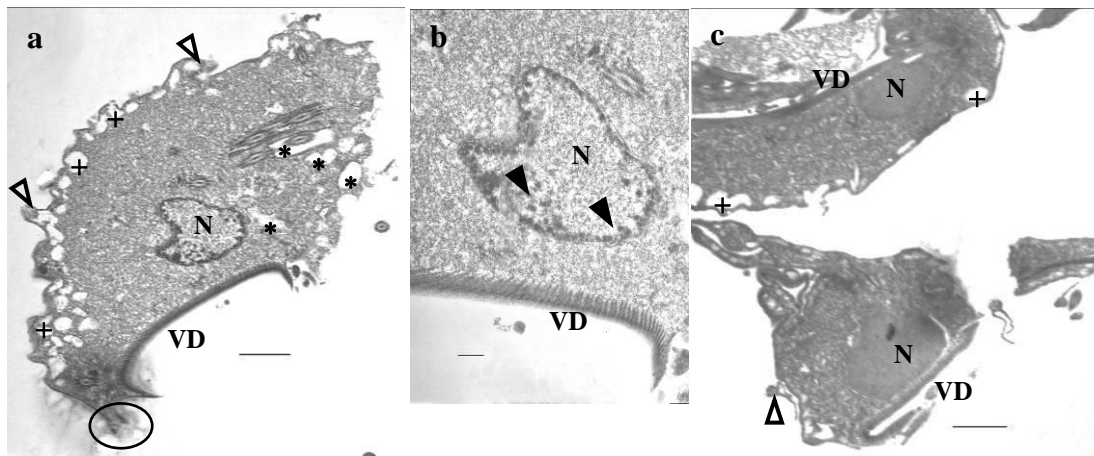
Figure 24 Transmission electron microscopic image showing *Giardia intestinalis* treated with 0.42 μg/ml metronidazole for 24 h. Note membrane blebs (open triangle), ventrolateral flange disruption (open circle), enlargement of PV (cross), empty spaces in cytoplasm (asterisks) and condensation of chromatin (black arrowheads). a and c, bars = 1 μm; b, bar 200 nm.
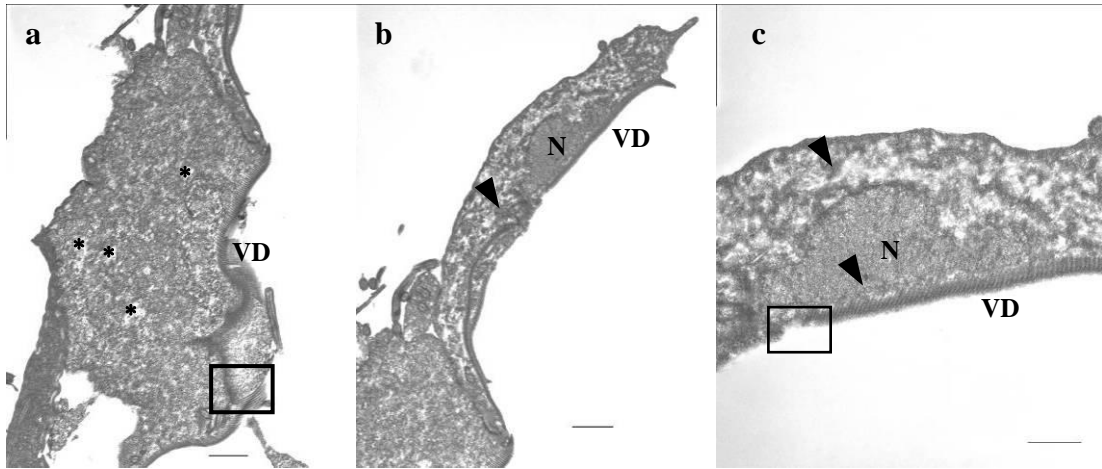
Figure 25 Transmission electron microscopic image showing *Giardia intestinalis* trophozoite treated with 0.42 µg/ml 1-hydroxy-2-hydroxymethylanthraquinone for 24 h. Note large vacuoles in the cytoplasm (asterisks), fragmentation of ventral disk (square), and presence of electrodense precipitates in cytoplasm and nuclei (black arrowheads). a and b, bars = 1 µm; c, bar 500 nm.

3.7   Mechanisms of 1-hydroxy-2-hydroxymethylanthraquinone against growth of *Giardia intestinalis*

3.7.1 Apoptosis assay

Annexin V and PI staining of *G. intestinalis* trophozoite viewed under bright field and fluorescence microscopy, respectively, are compared in Figure 26. Normal cells were negative for both annexin V and PI when viewed under fluorescence microscopy (Figure 26b), while red-fluorescent cells indicated necrosis stage (Figure 26d) and late stage of apoptotic cells demonstrated both annexin V and PI positive as shown in Figure 26f.

Figure 27-29 represented the histogram of forward scatter (FSC) and side scatter (SSC) of *G. intestinalis* trophozoite from flow cytometry analysis after being incubated in complete medium, IC_{50} of metronidazole and CFQ for 6, 12 and 24 h, respectively. The population of trophozoites treated metronidazole (Figure 27b) at 6 h is similar to that of the control (Figure 27a). In the other hand, *G. intestinalis* trophozoites treated with CFQ at IC_{50} concentration exhibit decrease in FSC and increase in SSC, since 6 h of incubation (Figure 27c) when compared with control (Figure 27a), indicated increase in apoptotic cell population. The percentage of early and late apoptotic *G. intestinalis* trophozoites (1.4% and 25.8%, respectively) treated with CFQ at 12 h (Figure 28c), were significantly higher than *G. intestinalis* trophozoites treated with control (Figure 28a) or metronidazole (Figure 28b). At 24 h of incubation, about 74.8% of *G. intestinalis* trophozoites treated in CFQ (Figure 29c) clearly in late apoptotic condition while 95.1% and 83.8% of the cells in complete medium and metronidazole, respectively, were found undamaged (Figure 29a and b, respectively).
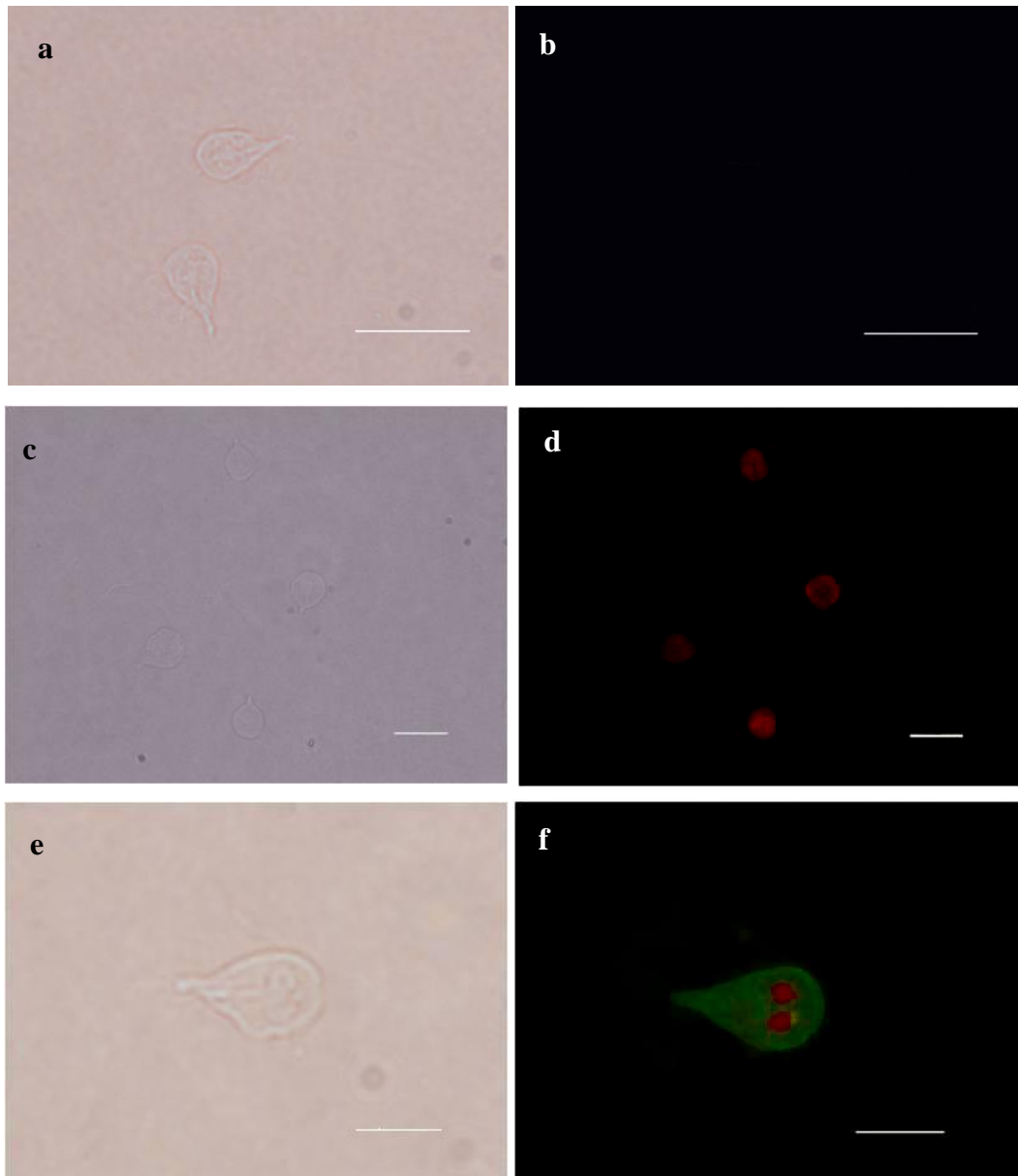
Figure 26 Images of *Giardia intestinalis* trophozoites after stained with annexin V and PI. (a, b) normal *Giardia intestinalis* trophozoite view under bright field and fluorescence microscopy, respectively (both annexin V and PI negative).

(c-f) *Giardia intestinalis* treated with 1-hydroxy-2-hydroxymethylanthraquinone 0.42 µg/ml for 24 h, trophozoites are stained PI only indicated necrosis of cell (c, d) and trophozoites stain both annexin V (green color) and PI (red) indicated late apoptotic cells (e, f).

Bars, a-d = 20 µm, e-f = 10 µm.
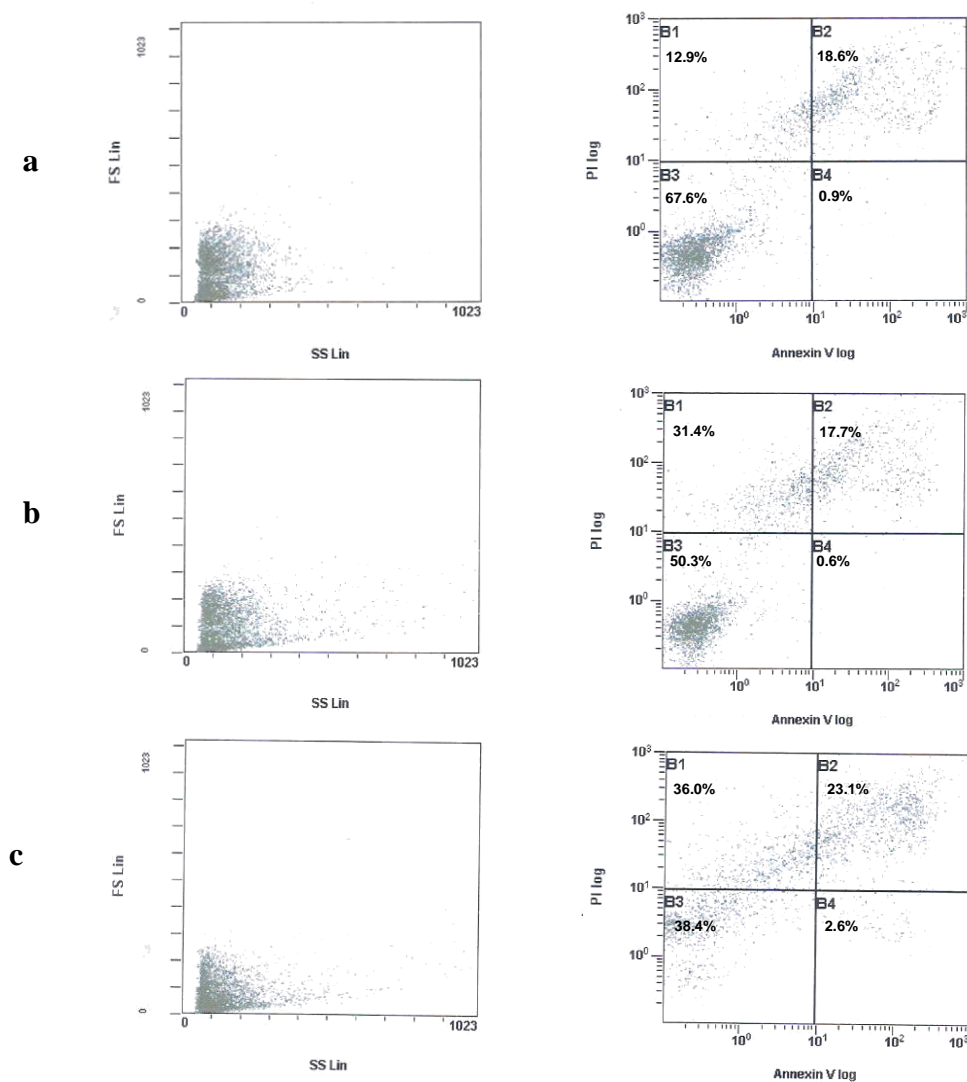
Figure 27 Dot plots of forward-angle light scatter vs. side-angle light scatter (left panel) and dot plot analysis of *Giardia intestinalis* trophozoite (right panel). The trophozoites were stained with annexin V-FITC and PI. Quadrant B3 (FITC$^-$/PI$^-$) indicated intact cells, B4 (FITC$^+$/PI$^-$) indicated apoptotic and nonviable cells, B2 (FITC$^+$/PI$^+$) indicated late apoptotic cells and B1 (FITC$^-$/PI$^+$) indicated necrotic cells. The percentage of each cell type is indicated in the relative quadrants after being incubated for 6 h in:

(a) complete medium,

(b) 0.42 μg/ml metronidazole

(c) 0.42 μg/ml 1-hydroxy-2-hydroxymethylanthraquinone.
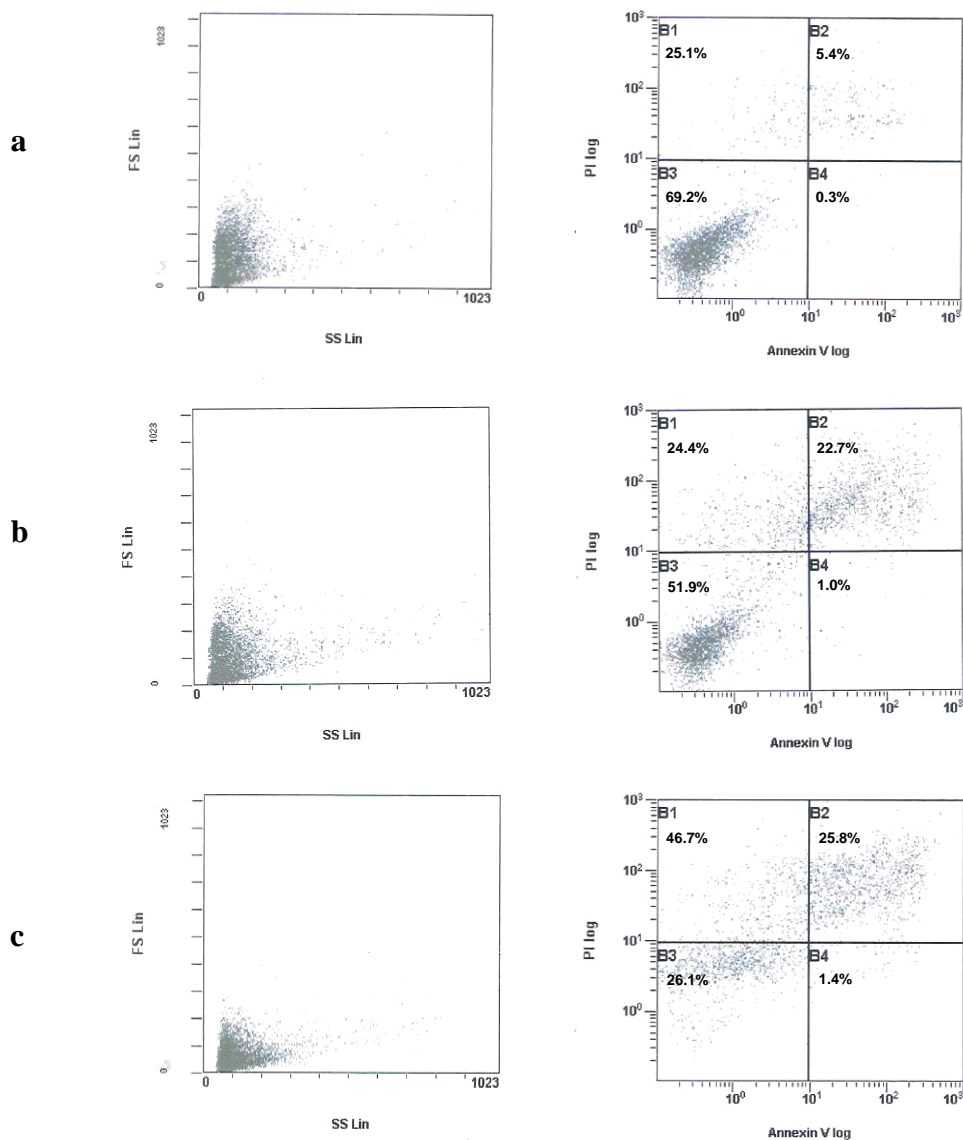
Figure 28 Dot plots of forward-angle light scatter vs. side-angle light scatter (left panel) and dot plot analysis of *Giardia intestinalis* trophozoite (right panel). The cells were stained with annexin V-FITC and PI. Quadrant B3 (FITC$^-$/PI$^-$) indicated intact cells, B4 (FITC$^+$/PI$^-$) indicated apoptotic and nonviable cells, B2 (FITC$^+$/PI$^+$) indicated late apoptotic cells and B1 (FITC$^-$/PI$^+$) indicated necrotic cells. The percentage of each cell type is indicated in the relative quadrants after being incubated for 12 h in:

(a) complete medium,

(b) 0.42 μg/ml metronidazole

(c) 0.42 μg/ml 1-hydroxy-2-hydroxymethylanthraquinone.
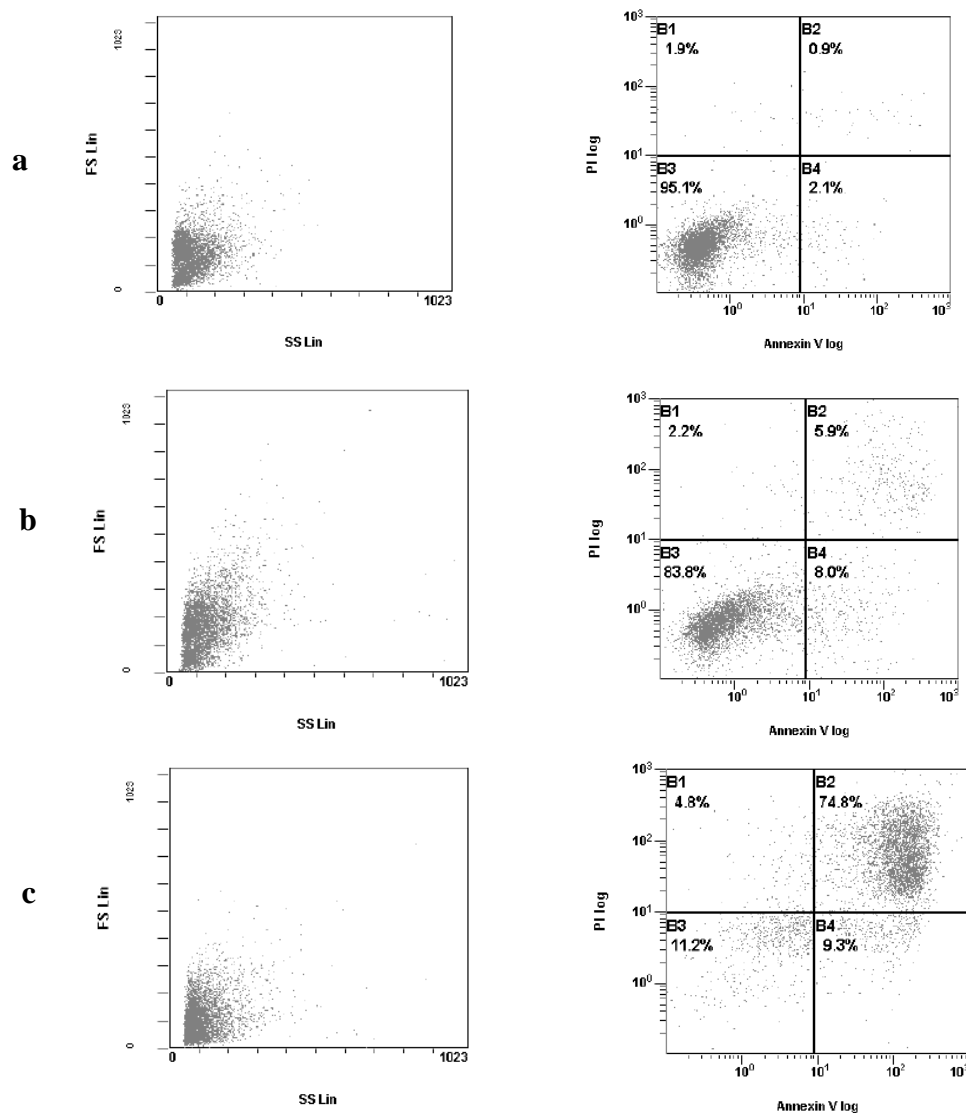
Figure 29 Dot plots of forward-angle light scatter vs. side-angle light scatter (left panel) and dot plot analysis of *Giardia intestinalis* trophozoite (right panel). The cells were stained with annexin V-FITC and PI. Quadrant B3 (FITC$^-$/PI$^-$) indicated intact cells, B4 (FITC$^+$/PI$^-$) indicated apoptotic and nonviable cells, B2 (FITC$^+$/PI$^+$) indicated late apoptotic cells and B1 (FITC$^-$/PI$^+$) indicated necrotic cells. The percentage of each cell type is indicated in the relative quadrants after being incubated for 24 h in:

(a) complete medium,

(b) 0.42 μg/ml metronidazole

(c) 0.42 μg/ml 1-hydroxy-2-hydroxymethylanthraquinone.

3.7.2 DNA fragmentation

Bright field and fluorescence photomicrographs of DNA in *G. intestinalis* trophozoite treated with IC$_{50}$ of metronidazole and CFQ for 24 h after stained by TUNEL assay are shown in Figure 30. In normal trophozoites, a small number of DNA fragmentations were observed (Figure 30a). On the other hand, *G. intestinalis* DNA after treated with metronidazole (Figure 30b) displayed only green fluorescence in the nucleus indicated early apoptosis and *G. intestinalis* treated with CFQ (Figure 30c) presented with condensed and fragmented orange chromatin clearly indicated late apoptotic.
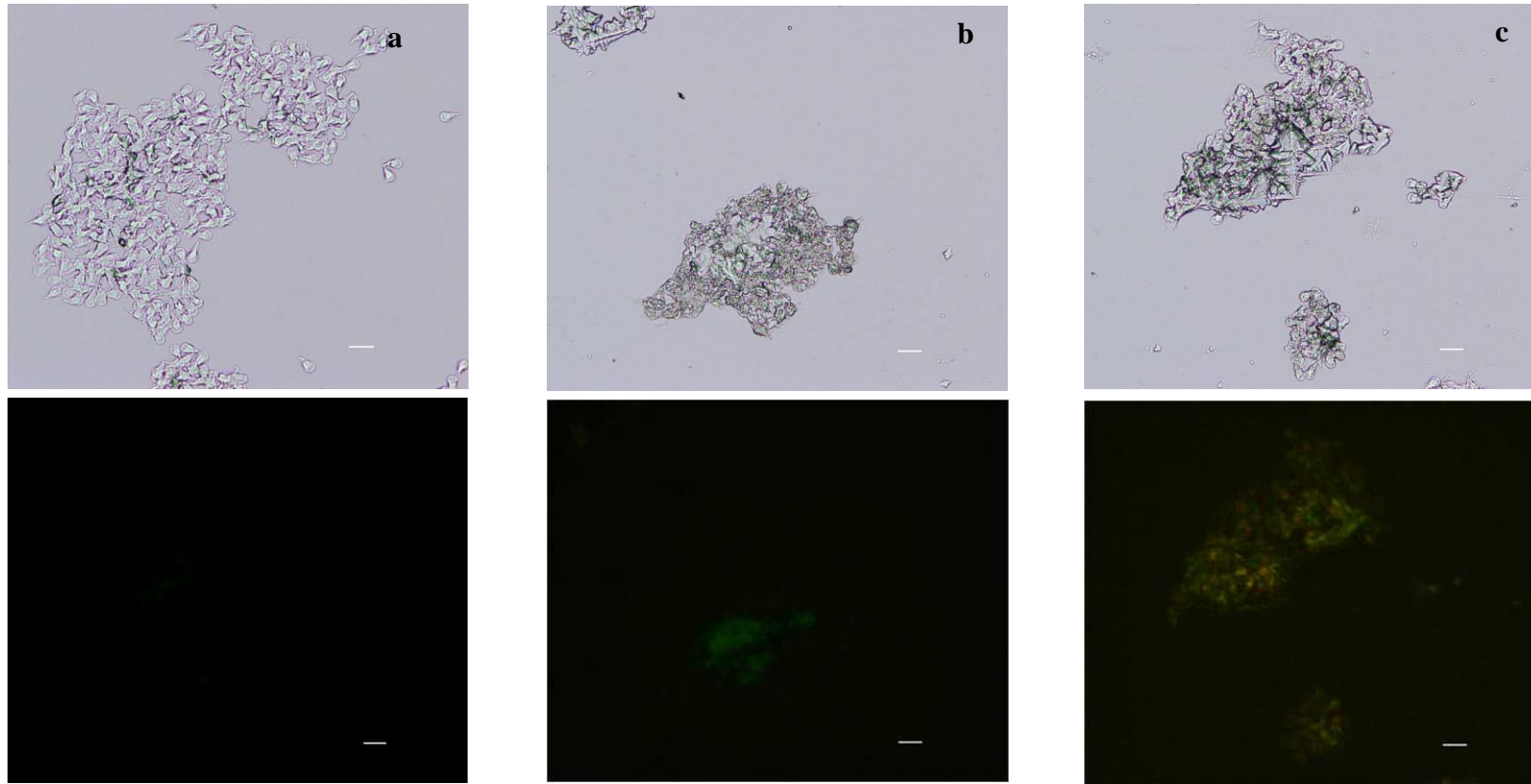
Figure 30 Bright field (upper panel) and fluorescence (lower panel) photomicrographs of *Giardia intestinalis* from TUNEL assay for in situ DNA fragmentation after being incubated for 24 h in:

(a) complete medium, (b) 0.42 μg/ml metronidazole (early apoptosis) and (c) 0.42 μg/ml 1-hydroxy-2-hydroxymethylanthraquinone (late apoptosis). Bars = 20 μm.

3.8   Cell cycle analysis

Figure 31, shows the images of normal *G. intestinalis* trophozoites after incubated with RNase and PI to confirm the red fluorescence stained in the nucleus when view under fluorescence microscope. The percentage of different phases of *G. intestinalis* treated with metronidazole and CFQ for 6, 12 and 24 h and analysed by flow cytometer are shown in Figure 32. The distribution of DNA trough cell cycle of *G. intestinalis* treated with metronidazole and CFQ were not significantly different from the control without drugs. The main peak of all experiments is in G1 phase follow by S and G2/M, respectively.
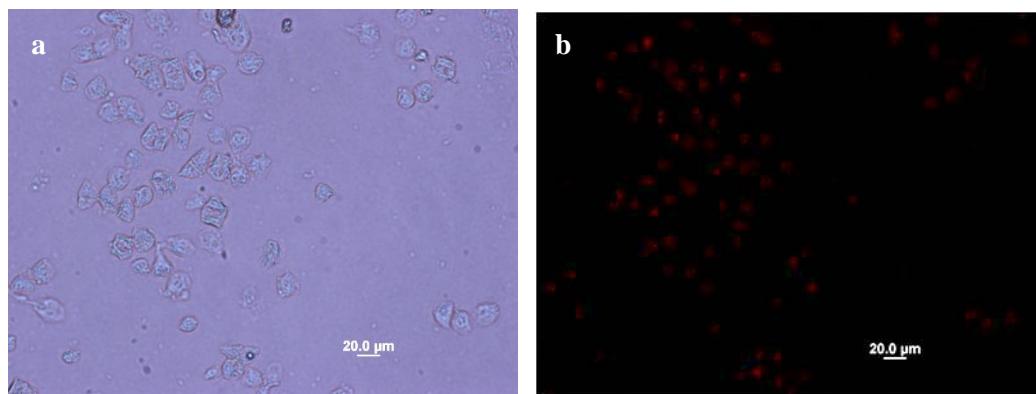
Figure 31 Bright field (a) and fluorescence (b) photomicrographs of untreated *Giardia intestinalis* trophozoite after 24 h incubation period with RNase and propidium iodide that showed red-fluorescent nuclear.
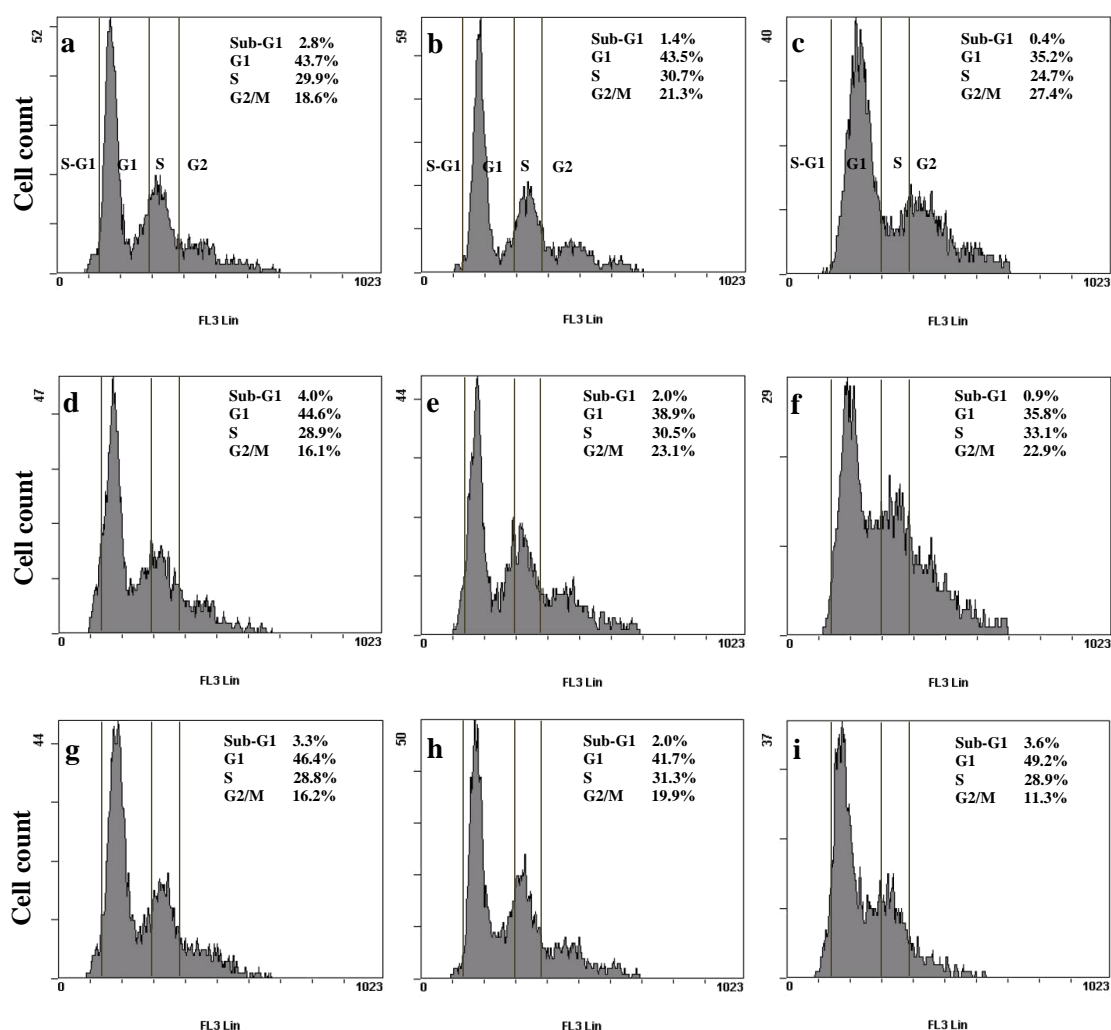
Figure 32 Histograms of cell cycle analysis of *Giardia intestinalis* trophozoite were analyzed by flow cytometry after propidium iodide staining. (a, b, c) Untreated control, (d, e, f) trophozoite were treated with IC_{50} of metronidazole and (g, h, i) trophozoite were treated IC_{50} of 1-hydroxy-2-hydroxymethylanthraquinone compound for 6, 12 and 24 h, respectively. S-G1, G1, S and G2 peaks are showing sub-G1, G1, S and G2/M phase in the trophozoite cell cycle.

3.9   Adhesion of *Giardia intestinalis* to Caco-2 cell line

Figure 33, 34 and 35 shows the attachment of *G. intestinalis* to Caco-2 cell line (black arrows) in 24-well plate after stained with 5% Giemsa stain and view under phase contrast microscope after incubated in complete medium, metronidazole or CFQ at IC_{50} concentration for 24 h, respectively. Percentage of *G. intestinalis* trophozoite attached to Caco-2 cell line at different time interval is given in table 6.

The percentages of *G. intestinalis* trophozoites attached to Caco-2 cell line were significantly ($p<0.05$) decreased start from as early as 6 h after being incubated in IC_{50} concentration of metronidazole and CFQ when compared to control. However, no significantly different between the percentages of *G. intestinalis* trophozoites attached to Caco-2 cell after treated with metronidazole or CFQ at all time interval.
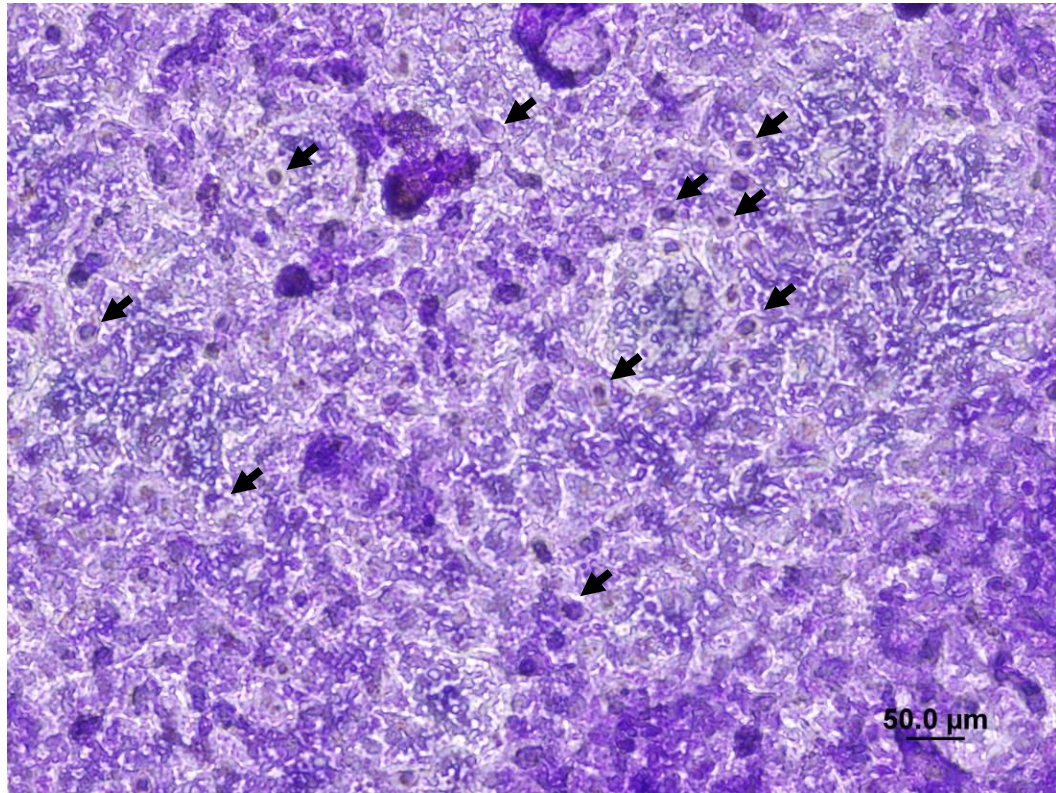
Figure 33 Phase contrast image of untreated *Giardia intestinalis* trophozoites (black arrows) co-cultured with Caco-2 cell line for 24 h and after stained with 5% Giemsa stain.
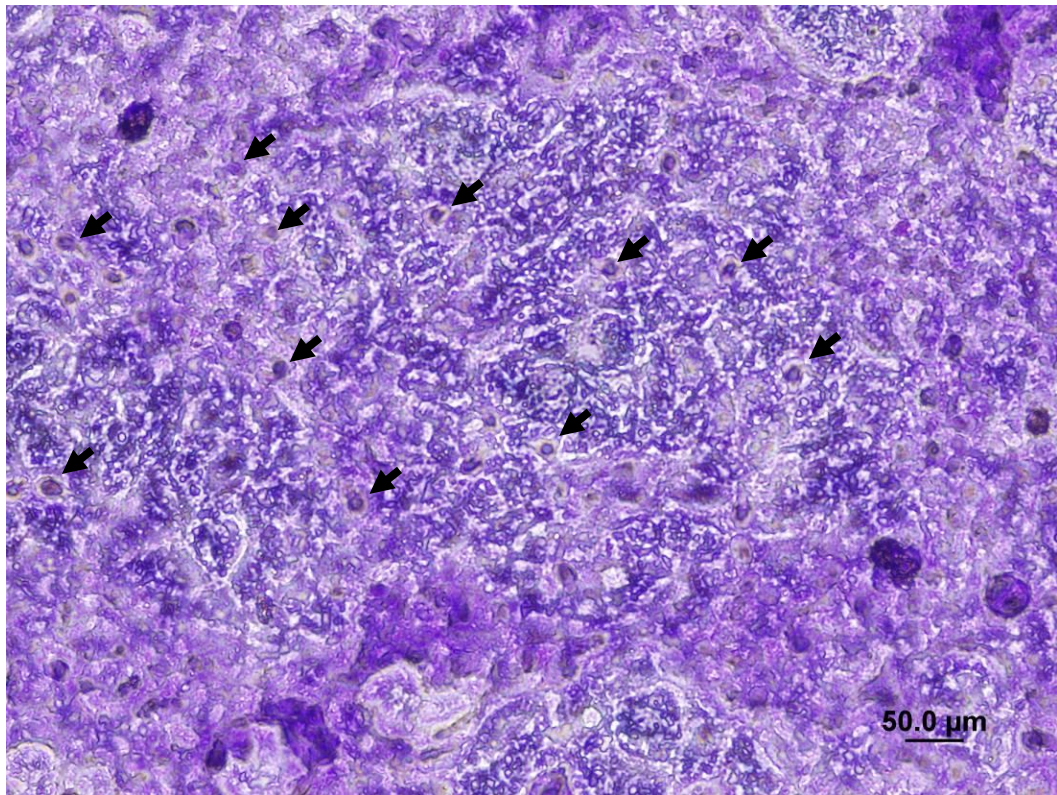
Figure 34 Phase contrast image of *Giardia intestinalis* trophozoites (black arrows) co-cultured with Caco-2 cell line treated with 0.42 µg/ml metronidazole for 24 h of incubation and after stained with 5% Giemsa stain.
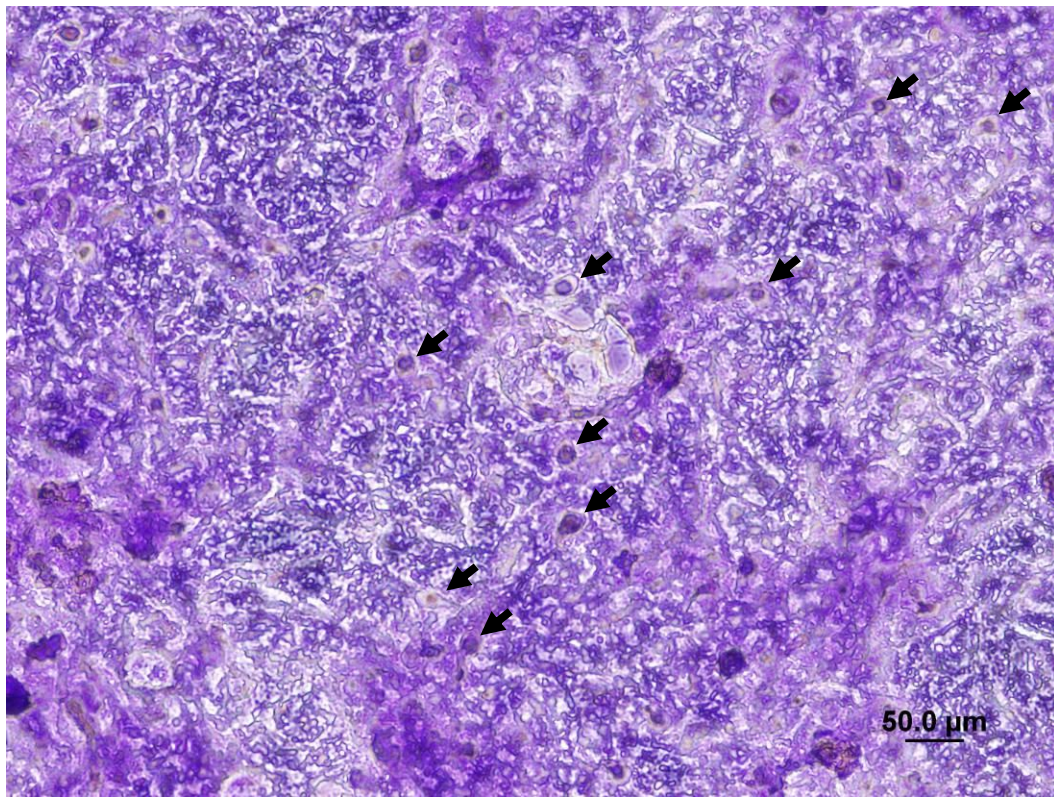
Figure 35 Phase contrast image of *Giardia intestinalis* trophozoites (black arrows) co-cultured with Caco-2 cell line treated with 0.42 µg/ml 1-hydroxy-2-hydroxymethylanthraquinone for 24 h and after stained with 5% Giemsa stain.

Table 6 Percentage of *Giardia intestinalis* trophozoites that attached to Caco-2 cell line after being incubated in IC$_{50}$ concentration of metronidazole or 1-hydroxy-2-hydroxymethylanthraquinone for 6, 12 and 24 h, respectively (mean ± standard error of the mean, n= 3).

Compounds	% Attachment		
	6 h	12 h	24 h
Control	100.00[a]	100.00[a]	100.00[a]
Metronidaozole (0.42 µg/ml)	86.19 ± 17.09[b]	80.26 ± 15.43[b]	39.59 ± 30.00[b]
CFQ (0.42 µg/ml)	70.73 ± 10.08[b]	70.36 ± 11.27[b]	46.23 ± 26.62[b]

[a,b] Different letters in the same column indicate significant differences ($p < 0.05$).

CHAPTER 4

DISCUSSION

4.1 Anti-intestinal protozoa from medicinal plants and CFQ compound

Gastrointestinal diseases such as dysentery caused by *Entamoeba histolytica* and diarrhea caused by *Giardia intestinalis* are the major health problem worldwide. Nitroimidazoles drugs (usually metronidazole) are being used for the treatment of amobiasis and giardiasis. However, this drug is reported to have potential carcinogenic and unpleasant side effects (Gardner and Hill, 2001; Wright et al., 2003). Therefore, as an alternate to available nitroimidazoles drugs, the present work was aimed in search for anti-*Entamoeba* and anti-*Giardia* agents from Thai medicinal plants.

A total of 23 extracts from 21 Thai medicinal plants (Table 2) were investigated for their anti-protozoal activities. It was found that seven plant extracts including *C. flavescens*, *C. longa*, *E. thymifolia*, *G. mangostana*, *P. granatum*, *S. koetjape* and *T. bellerica* which exhibited both anti-amoebic and anti-giardial activities *in vitro* at concentration ranging from 15.63-500 μg/ml. All the seven active plants were claimed to have anti-diarrhoeal activity in Thai traditional medicine. These findings were in agreement with some other reports such as compounds from *C. longa* and *P. granatum* which were found to inhibit *E. histolytica* or *G. intestinalis* (Chattopadhyay et al., 2004; Calzada et al., 2006; Perez-Arriaga et al., 2006; Haddad et al., 2011).

In the present study, acetone extract from *C. flavescens* is the most effective against both *E. histolytica* and *G. intestinalis* growth *in vitro*, with MICs of 125 and 15.63 μg/ml, respectively. *C. flavescens* is a Thai traditional plant that is used in local people in Satun province to expel worm or parasites from children (Madardam, 2010). This plant is hardwood vine, elliptical shape leaf with relatively

thin blade. Its flowers are fragrant, whitish or creamy white to pale yellow, 5-merous and are clustered in groups (http://homepage.univie.ac.at/christian.puff/_FTH-RUB/FTH-RUB_HOME.htm). The acetone extract from this plant was also reported to have moderate activity against methicillin-resistant *Staphylococcus aureus* with MIC value of 128 μg/ml (Kongyen et al., 2014) and this is the first report on the activity of *C. flavescens* extract in inhibiting the growth of *E. histolytica* and *G. intestinalis*.

Curcuma longa (turmeric), a member of the family Zingiberaceae (ginger), is extensively cultivated and used in several food ingredients in tropical countries such as China, India, Thailand etc. In the present work, the extract from *C. longa* was active against both *E. histolytica* and *G. intestinalis* at MIC of 250 μg/ml. *C. longa* extract has been reported to act against several other parasites such as *E. histolytica*, *Plasmodium*, *Leishmania*, *Trypanosoma* and *Schistosoma* (Chattopadhyay et al., 2004). *Acanthamoeba castellanii* cyst was also inhibited by ethanol extract of *C. longa* at 100 mg/ml (El-Sayed et al., 2012). Curcumin, a major bioactive compound from *C. longa*, exhibited anti-inflammatory, anti-*Leishmania*, anti-carcinogenic, anti-oxidant, anti-mutagenic, anti-diabetic, anti-bacterial, anti-fungal and anti-viral activities (Koide et al., 2002; Chattopadhyay et al., 2004; Haddad et al., 2011). The activity of curcumin on *G. intestinalis* included inhibition of trophozoite adherent capacity, induced morphological alterations and provoked apoptosis (Perez-Arriaga et al., 2006). Moreover, the essential oil of *C. longa* inhibited the growth of several pathogenic bacteria (Peret-Almeida et al., 2008).

Punica granatum or pomegranate is a bush plant with worldwide application in folk medicine. Different parts of pomegranate have been known as a reservoir of bioactive compounds with potential biological activities e.g. anti-cancer (Nair et al., 2011), anti-oxidant (Chidambara et al., 2002; Triqueros et al., 2014), anti-fungal (Foss et al., 2014), anti-schistosomal and anti-helminthic (Fahmy et al., 2009). In addition, Reddy et al. (2007) have reported that *P. granatum* exhibited anti-microbial, anti-oxidant, and anti-plasmodial activities. In this work, it was found that the *P. granatum* extract showed the MIC values against *E. histolytica* and *G. intestinalis* of 500 and 250 μg/ml, respectively. These results are in line with report of

Calzada et al. (2006) that presented the activity of *P. granatum* extract which inhibited both *E. histolytica* and *G. intestinalis* with the IC_{50} value of 29.5 and 198.5 µg/ml, respectively.

Euphorbia thymifolia is commonly used in folk medicine in Bangladesh for the treatment of helminthiasis (Kane et al., 2009; Rahmatullah et al., 2012), and was found to possess anti-viral and anti-oxidant activities (Lin et al., 2002). Furthermore, this plant is traditionally used as astringent in diarrhea, dysentery, haemostatic, sedative and anti-helminthic etc. (Mali and Panchal, 2013). Alkaloids from *E. thymifolia* exhibited anti-bacterial activity against *Bacillus subtilis* ATCC-6633, *Escherichia coli* ATCC-8739, *Klebsiella pneumonia* AYCC-10031, *Staphylococcus aureus* ATCC-25923, and anti-fungal activity against *Candida albicans* (Mali and Panchal, 2013). This is the first report of an extract from *E. thymifolia* that inhibited *E. histolytica* and *G. intestinalis in vitro*.

Garcinia mangostana is a plant growing well in the tropical areas including Thailand, Indonesia, Philippines, and Malaysia. There are several bioactive compounds such as triterpenoids, xanthones, flavonoids and benzophenones, which have been isolated, and identified (Chin and Kinghorn, 2008). The pericarps of *G. mangostana* have been widely used as a traditional medicine for the treatment of diarrhoea, inflammation, skin infections and chronic wounds in South East Asia for many years (Mahabusarakam et al., 1987; Pedraza-Chaverri et al., 2008; Gutierrez-Orozco and Failla, 2013). Moreover, medicinal properties of *G. mangostana* extract e.g. anti-oxidant, anti-tumor, anti-inflammatory, anti-allergy, anti-malarial, pro-apoptotic, anti-cancer and anti-bacterial/viral properties have been reported (Pedraza-Chaverri et al., 2008; Obolskiy et al., 2009; Shan et al., 2011; Aisha et al., 2012). We further added more value to a *G. mangostana* extract as it also exhibited moderate anti-*E. histolytica* and anti-*G. intestinalis* activities.

An aqueous extract of *S. koetjape* bark is used traditionally in Malaysia as a tonic after giving birth (Rasadah et al., 2004). The biological and phamarcological properties of pure compounds isolated from different parts of *S. koetjape* have been reported to possess anti-viral (Wiart, 2006), anti-inflammatory (Rasadah et al., 2004) and anti-cancer activities (Kaneda et al., 1992). The roots of *S.*

koetjape are used to treat intestinal disorders throughout Southeast Asia (Wiart, 2006). The present work for the first time reports its anti-*E. histolytica* and anti-*G. intestinalis* activities.

Terminalia chebula is used as a remedy against a sore throat and cough, against diarrhoea connected to a prolapsed rectum, against ulcers and dysentery in China and Tibet (Singh et al., 2012). It is also found in Thailand (Kusirisin et al., 2009), Pakistan (Saleem et al., 2001), and India (Nariya et al., 2009). The fruits of this plant are rich in tannin content about 32%. The powder of *T. chebula* fruits has been used to treat chronic diarrhoea (Chattopadhyay and Bhattacharyya, 2007; Bag et al., 2013) and amobiasis (Sohni et al., 1995). *T. chebula* is considered as a natural remedy for rejuvenation, wound healing, and skin diseases in traditional Thai medicine (Chulasiri et al., 2011). Furthermore, it is effective against bacteria (Kim et al., 2006) that included *Helicobacter pylori* (El-Mekkawy et al., 1995), *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus* (Kumar et al., 2008). We reported here its anti-giardial activity.

Three pure compounds were isolated from *C. flavescens* and their anti-protozoal activities were evaluated. Only 1-hydroxy-2-hydroxymethylanthraquinone (CFQ) exhibited anti-amoeba (MIC = 20 µg/ml) and anti-giardial (MIC = 2.5 µg/ml) activities. It was also moderately active (MIC = 32 µg/ml) against methicillin-resistant *Staphylococcus aureus* (Kongyen et al., 2014). In time killing assay, the percentage viability of *E. histolytica* trophozoite at MIC concentration (20 µg/ml) of CFQ, was comparable to 2.5 µg/ml metronidazole (Figure 12). On the other hand, at a similar MIC concentration (2.5 µg/ml for both metronidazole and CFQ), the percentage viablility of *G. intestinalis* trophozoite exposed to CFQ significantly ($p<0.05$) lower than when exposed to metronidazole within 12 h of incubation (Figure 13). These results have indicated that, at the same concentration, CFQ from *C. flavescens* can inhibit *G. intestinalis* faster than metronidazole. This would be one advantage of its future therapeutic use as anti-*Giardia* infection. CFQ is an anthraquinone derivative and has been isolated from rhizome of *Morinda parvifolia* (Chang and Lee, 1984) and root of *Damnacanthus indicus* (Yang et al., 1992). However, its bioactivity has not been studied. Anthraquinones from *Cassia* species

are known to possess anti-fungal, anti-plasmodial, anti-inflammatory and anti-diarrhoeal activities (Dave and Ledwani, 2012).

As the mechanism of metronidazole and CFQ at their IC_{50} concentration were compared under SEM, *E. histolytica* trophozoite treated with 4.59 µg/ml and *G. intestinalis* trophozoites treated with 0.42 µg/ml CFQ for 24 h showed evidence of abnormal trophozoite morphology (Figure 16 and 19) characterized by holes on their surface to indicate that they had lost membrane function. Upon morphological alteration, the disassemble of *G. intestinalis* sucking disc or rough cell surface and membrane bleb in *E. histolytica* may induce trophozoites to detach from the host; destruction on the surface of the plasma membrane eventually caused parasite to loss their ability to survive.

The morphology of *G. intestinalis* after exposed to CFQ under SEM was similar to the observations of Machado et al. (2010; 2011) who studied the effect of essential oil from *Thymbra capitata*, *Origanum virens*, *Thymus zygis* subsp. *sylvestris*, *Lippia graveolens* and *Syzygium aromaticum* against *G. intestinalis*. CFQ alters integrity of adhesive disk of the trophozoites, and causes damage to the plasma membrane (Figure 25). The lost of membrane continuity may cause *G. intestinalis* to undergo cell apoptosis as indicated by the finding annexin V green fluorescence bound to phosphatidylserine that was located on the outer leaflet of the plasma membrane (Figure 26f).

The results of TEM analysis were in agreement with the observation by SEM. TEM of both metronidazole and CFQ treated *E. histolytica* showed an increasing in the size and number of vacuoles and glycogen content in cytoplasm with electron condensation in nucleus (Figure 21 and 22). This phenomenon indicated apoptotic character. Similar morphological alterations were also observed in *E. histolytica* treated with IC_{50} concentration of 0.017 µg/ml nitazoxanide (Cedillo-Rivera et al., 2002) or 10 µg/ml aminoglycoside antibiotic G418 (Villalba et al., 2007). Under TEM, *G. intestinalis* clearly showed nuclear condensation, cell shrinkage, deposition of membrane-bound apoptotic bodies, and heavy vacuolization all of which indicated the characteristics of apoptosis. These morphological alterations emphasize the blockage of trophozoites adherence and loss of viability.

Following SEM and TEM analysis, it was concluded that the concentration CFQ use to react against *E. histolytica* was high when compare to metronidazole or other compound found in literature, ie. nitazoxanide ($IC_{50} = 0.017$ µg/ml) (Cedillo-Rivera et al., 2002), emetine ($IC_{50} = 1.05$ µg/ml) and *Chiranthodendron pentadactylon* extract ($IC_{50} = 2.5$ µg/ml) (Calzada et al., 2006). Thus, the effect of metronidazole and CFQ on the adherence, apoptosis, DNA fragmentation and cell cycle of *G. intestinalis* were further studied.

In our approach to elucidate the mechanism of CFQ from *C. flavescens* against *G. intestinalis* trophozoite, it was found that the IC_{50} concentration of CFQ at 0.42 µg/ml was sufficient to induce apoptosis in a majority of *G. intestinalis* trophozoites (74.8%) within 24 h as shown by flow cytometry (Figure 29c). In contrast, after being exposed to the IC_{50} concentration of metronidazole, most *G. intestinalis* trophozoites (83.6%) remained intact (Figure 29b). However, the trophozoites were dead as was clearly shown by the SEM (Figure 18) with a round shape, an irregular dorsal surface and a ventral disk with membrane blebs. In our study, we found a slight apoptosis caused by metronidazole when compared to control and CFQ. It is likely that the concentration of metronidazole used in our study (0.42 µg/ml) was too low even though this was the IC_{50} value. Metronidazole (40 µg/ml) induction of apoptosis in *G. intestinalis* had demonstrated by Sandhu et al. (2004), also by Ghosh et al. (2009) who used 1 µg/ml and by Bagchi et al. (2012) who used 2 and 5 µg/ml. Protozoan parasites, *Blastocystis hominis*, do undergo apoptosis after exposure to metronidazole at a concentration of 0.5 µM (Nasirudeen et al., 2004). The mechanism of metronidazole action against *G. intestinalis* includes its conversion to a toxic compound from reduction products when it was reduced by pyruvate: ferredoxin oxidoreductase (Wright et al., 2003; Watkins and Eckmann, 2014) and caused DNA damage by DNA fragmentation (Harris et al., 2001). In *E. histolytica* (Leitsch et al., 2007), and *Trichomonas vaginalis* (Leitsch et al., 2009), metronidazole bound to proteins involved in the thioredoxin-mediated redox network and disrupted the redox equilibrium by inhibiting thioredoxin reductase and depleting intracellular thiol pools. These may be some reasons to explain the presence of intact trophozoites detected by flow cytometer when treated with 0.42 µg/ml metronidazole until after 24 h. Several

plant extracts have been found to induce apoptosis in protozoa for instance, curcumin from *C. longa* on *G. intestinalis* (Perez-Arriaga et al., 2006), essential oil from *Cymbopogon citratus* and *Allium sativum* on *Leishmania* spp. (Khademvatan et al., 2011; Machado et al., 2012). Moreover, *Voacanga globosa* (Vital et al., 2010), *Ficus septica* and *Sterculia foetida* leaf extracts also induced apoptosis in *Trichomonas vaginalis* and *E. histolytica* (Vital et al., 2010).

TUNEL assay for in situ DNA fragmentation after being incubated *G. intestinalis* with CFQ for 24 h confirmed that trophozoites were mainly in their late apoptotic stage as shown by condensed and fragmented orange chromatin (Figure 30c) while only green fluorescence was detected in the nucleus when treated with metronidazole (Figure 30b). The principle of TUNEL staining is based on fluorochrome-labeling of 3′-OH ends of DNA strand breaks, catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT). This method allows the discrimination of apoptosis and specifically labels DNA strand breaks generated during apoptosis, which might be consequences of activation of apoptotic mechanisms that may be exclusive for microorganisms lacking mitochondria (Perez-Arriaga et al., 2006). These results were supported by histogram of flow cytometry after annexin V and PI staining in case of treated metronidazole (early apoptosis in Figure 29b) and treated CFQ (late apoptosis in Figure 29c) at IC_{50} concentration for 24 h.

Efficient growth and differentiation is an important step for *G. intestinalis* to infect a host and cause disease. During normal cell cycle the binucleate trophozoites cycle between a cellular ploidy of 4N and 8N and during stationary phase trophozoites arrest in G2 phase with a ploidy of 8N (Bernander et al., 2001; Reiner et al., 2008). In our study, no significant different of cell cycle were found in *G. intestinalis* trophozoite after being treated in IC_{50} concentration of metronidazole or CFQ for 24 h. However, the work of Sandhu et al. (2004) has indicated that metronidazole at 40 µg/ml arrest growth in S phase of *G. intestinalis* cell cycle. The drug, metronidazole, is known to exert its toxic effect through unstable intermediate products (nitro and nitroso-free radicals) (Ellis et al., 1992). DNA damage, stand breakages, cross-linking in the nucleic acid and the release of nucleotide phosphates are consequent of the reactive products that interact with DNA. Previous study of

Hoyne et al. (1989) has demonstrated that furazolidone inhibited the cell cycle causing an arrest in the S and G2/M phases while metronidazole arrested cell cycle progression in the G2/M phase. Though several reports have showed the effect of the drug on cell cycle in S and G2/M phase, our work presents no different in each phase of cell cycle probably due to low concentration of the compound used.

The attachment of *G. intestinalis* to intestinal mucosa is an important factor for giardiasis pathogenesis as well as for their survival and colonization in the host. The adherence assay showed that both metronidazole and CFQ caused significant detachment of trophozoites from Caco-2 cell line. Both metronidazole and CFQ rendered the trophozoites unable to attach to the host cell which is a prerequisite for establishment of *Giardia* infection.

In conclusion, CFQ may be considered to be a new drug active against *G. intestinalis* infection as it causes alterations of trophozoite morphology and cell membrane rupture leading to late apoptosis of the cell as well as the detachment of trophozoite to Caco-2 cell line.

CHAPTER 5

CONCLUSIONS

In the present work, we have tested 23 extracts from 21 Thai medicinal plants against *in vitro* growth of *Entamoeba histolytica* and *Giardia intetinalis*. The crude acetone extract from *Coptosapelta flavescens* exhibited the best activity against *in vitro* growth of *E. histolytica* and *G. intestinalis* (MIC = 125 and 15.63 µg/ml, respectively) while the MIC value of metronidazole against both protozoa was 2.5 µg/ml. Two anthaquinones and one naphthoquinone were purified from *C. flavescens*. Only 1-hydroxy-2-hydroxymethylanthraquinone (CFQ) displayed the most growth inhibitory activity against both *E. histolytica* and *G. intestinalis* trophozoites with the IC_{50} value at 4.59 and 0.42 µg/ml, respectively. In time kill assay, after expose to CFQ, the percentage viability of *G. intestinalis* was lower than when exposed to a standard drug, metronidazole within 12 h incubation. While for *E. histolytica*, CFQ activity was comparable with metronidazole. Several morphological changes were found when incubated *E. histolytica* and *G. intestinalis* with IC_{50} concentration of CFQ as revealed by SEM and TEM. Further study revealed the mechanism of CFQ against *G. intestinalis* included induced apoptosis, morphology alteration, DNA fragmentation and reduced trophozoites adhesion to Caco-2 cells line.

This study suggested that CFQ isolated from *C. flavescens* seems to be a good candidate for use against amoeba and giardial infections. The effects of CFQ on animals and human should be further investigated before being use as anti-protozoan parasite in human.

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APPENDIX A

Medium preparation

1. Axenic culture media for *Giardia intestinalis* (Diamond et al., 1995)

1.1 YI broth preparation

Dissolved in this order in 600 ml distilled water:

- K$_2$HPO$_4$	1	g
- KH$_2$PO$_4$	0.6	g
- NaCl	1	g
- Yeast extract	30	g
- Glucose	10	g
- L-cysteine hydrochloride	2	g
- Ascorbic acid	0.2	g
- Ferric ammonium citrate	22.8	mg
- Dehydrated bovine bile	500	mg

Bring final volume to 880 ml with distilled water and pH to 7.0-7.1 using 1N sodium hydroxide solution and filtrated by membrane filter (0.45 μm). Dispense in 88 ml amount into 100 ml glass bottles. The sterile YI base can be stored frozen at -20° C for several months.

2. Axenic culture media for *Entamoeba histolytic* (Diamond et al., 1995)

2.1 YI-S broth preparation

Dissolved in this order in 600 ml distilled water:

- K$_2$HPO$_4$	1	g
- KH$_2$PO$_4$	0.6	g

- NaCl	1	g
- Yeast extract	30	g
- Glucose	10	g
- L-cysteine hydrochloride	2	g
- Ascorbic acid	0.2	g
- Ferric ammonium citrate	22.8	mg
- Dehydrated bovine bile	500	mg

Bring final volume to 880 ml with distilled water and pH to 6.8 using 1N sodium hydroxide solution. Dispense in 88 ml amount into 100 ml glass bottles and autoclave for 15 minutes at 121° C with 15 lbs. pressure. The sterile TYI base can be stored frozen at -20° C for several months.

3. Vitamin mixture #18 preparation
Solution 1

A	Niacinamide	45	mg
	Pyridoxal hydrochloride	4	mg
	Calcium pantothenate	23	mg
	Thianium hydrochloride	5	mg
	Vitamine B12	1.2	mg
	Bring final volume to 25 ml with distilled water		
B	Riboflavin	7	mg
	Bring final volume to 45 ml with distilled water		
C	Folic acid	5.5	mg
	Bring final volume to 45 ml with distilled water		
D	d-Biotin	2	mg
	Bring final volume to 45 ml with distilled water		

Solution 2

DL-6,8 thioclic acid	1	ml
95% ethanol	5	ml
Tween 80	500	mg

Bring final volume to 20 ml with distilled water

Combine solutions 1 and 2, bring the final volume to 200 ml with distilled water and sterilize through a 0.22 μm filter. Store in 10 ml amounts at 4°C for up to 6 months. The solution is light sensitive. The above recipe provides enough vitamin mix #18 to make ten liters of complete TYI-S-33.

4. Bovine serum and fetal bovine serum

Heat inactivated adult bovine serum and fetal bovine serum at 56°C for 30 min and aliquot 10 ml amount in 15 ml tube and stored at -20°C.

5. Complete medium

To complete YI or YI-S medium for use in axenic cultivation add 2.0 ml of vitamin mix #18 and 10 ml of heat inactivated adult bovine serum to each 88 ml of YI or YI-S broth. Use complete medium within 7 days.

6. Minimal Essential Medium (MEM)

MEM powder was dissolved in 600 ml distilled water and added 2.2 g of powdered sodium bicarbonate, cell culture grade, stir until dissolved. While stirring, adjust the pH to 7.2 of the medium by using 1N HCl or 1N NaOH added drop wise to correct pH. After that pour into clean volumetric flask and added distilled water to bring the solution to 1000 ml. The solution was mixed thoroughly by pouring back into beaker and sterilized immediately by filtration using a membrane with a pore size of 0.22 μm. Add 20 ml of heat inactivated fetal bovine serum and 1 ml of penicillin-streptmycin to the MEM solution and store liquid medium by refrigerating at 0-5 °C.

APPENDIX B

Table A Scores for *Giardia intestinalis* growing *in vitro* after incubation with plant extracts for 24 h**.**

No	Name	Code	Part used	Extraction solvent	Concentrations (µg/ml) 500				250				125				62.5				31.25				15.625				7.8125				3.90625			
					Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2	
					R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
1	*Aegel marmelos* (L.) Corr.	มะตูมแก่	fruit	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
2	*Ardisia colorata* Roxb.	พิลังกาสา	wood	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
3	*Centella asiatica* (L.) Urb.	บัวบก	whole plant	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
4	*Coptosapelta flavescens*	CMeOH	whole plant	Mt	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
		Cact	whole plant	Ace	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	2+	2+	2+	2+	2+	2+	2+	2+
5	*Curcuma longa* L.	ขมิ้น	rhizome	Et	1+	1+	1+	1+	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
6	*Derris scandens* Roxb. Benth.	เถาวัลย์เปรียง	stem	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
7	*Euphorbia thymifolia* L.	นมราชสีห์เล็ก	whole plant	Et	1+	1+	1+	1+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
8	*Garcinia mangostana* L.	มังคุด	skin	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
9	*Holarrhena pubescens* Wall. ex G. Don	โมกหลวง	bark	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
10	*Manilkara achras* (Mill.) Fosberg	ละมุด	fruit	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
11	*Morinda citrifolia* L.	ขอ	fruit	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

Table A (cont.) Scores for *Giardia intestinalis* growing *in vitro* after incubation with plant extracts for 24 h**.**

No	Name	Code	Part used	Extraction solvent	500				250				125				62.5				31.25				15.625				7.8125				3.90625			
					Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2		Time 1		Time 2	
					R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
12	*Peltophorum pterocarpum* (DC.) Backer. ex K.Heyne.	นนทรี	bark	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
13	*Piper aurantuacum*	ชะพลู	leaf	Et	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
14	*Piper betle* L.	พลู	leaf	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
15	*Piper chaba* Vahl.	ดีปลี	fruit	Et	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
16	*Psidium guajava* L.	ฝรั่ง	leaf	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
17	*Punica granatum* L.	ทับทิม	skin	Et	1+	1+	1+	1+	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
18	*Rhizophora mucronata* Poir	โกงกาง	bark	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
19	*Sandoricum koetjape* (Burm. f.) Merr.	กระท้อน	root	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
20	*Terminalia bellerica* (Gaertn.) Roxb.	สมอภิเภก	fruit	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
21	*Terminalia chebula* Retz.	สมอไทย	fruit	Et	1+	1+	1+	1+	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

Concentrations (µg/ml)

R1 = replicate 1, R2 = replicate 2

Et = ethanol, Mt = methanol, Ace = acetone

(Upcroft & Upcroft, 2001)	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 parasite 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)

Table B Scores for *Entamoeba histolytica* growing *in vitro* after incubation with plant extracts for 24 h.

No	Name	Code	Part used	Extraction solvent	500 Time 1 R1	500 Time 1 R2	500 Time 2 R1	500 Time 2 R2	250 Time 1 R1	250 Time 1 R2	250 Time 2 R1	250 Time 2 R2	125 Time 1 R1	125 Time 1 R2	125 Time 2 R1	125 Time 2 R2	62.5 Time 1 R1	62.5 Time 1 R2	62.5 Time 2 R1	62.5 Time 2 R2	31.25 Time 1 R1	31.25 Time 1 R2	31.25 Time 2 R1	31.25 Time 2 R2	15.625 Time 1 R1	15.625 Time 1 R2	15.625 Time 2 R1	15.625 Time 2 R2	7.8125 Time 1 R1	7.8125 Time 1 R2	7.8125 Time 2 R1	7.8125 Time 2 R2	3.90625 Time 1 R1	3.90625 Time 1 R2	3.90625 Time 2 R1	3.90625 Time 2 R2
1	*Aegel marmelos* (L.) Corr.	มะตูมแก่	fruit	Et	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
2	*Ardisia colorata* Roxb.	พิลังกาสา	wood	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
3	*Centella asiatica* (L.) Urb.	บัวบก	whole plant	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
4	*Coptosapelta flavescens*	CMeOH	whole plant	Mt	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
		Cact	whole plant	Ace	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
5	*Curcuma longa* L.	ขมิ้น	rhizome	Et	1+	1+	1+	1+	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
6	*Derris scandens* Roxb. Benth.	เถาวัลย์เปรียง	stem	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
7	*Euphorbia thymifolia* L.	นมราชสีห์เล็ก	whole plant	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
8	*Garcinia mangostana* L.	มังคุด	skin	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
9	*Holarrhena pubescens* Wall. ex G. Don	โมกหลวง	bark	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
10	*Manilkara achras* (Mill.) Fosberg	ละมุด	fruit	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
11	*Morinda citrifolia* L.	ขอ	fruit	Et	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

Table B (cont.) Scores for *Entamoeba histolytica* growing *in vitro* after incubation with plant extracts for 24 h**.**

	No	Name	Code	Part used	Extraction solvent	500 Time 1 R1	500 Time 1 R2	500 Time 2 R1	500 Time 2 R2	250 Time 1 R1	250 Time 1 R2	250 Time 2 R1	250 Time 2 R2	125 Time 1 R1	125 Time 1 R2	125 Time 2 R1	125 Time 2 R2	62.5 Time 1 R1	62.5 Time 1 R2	62.5 Time 2 R1	62.5 Time 2 R2	31.25 Time 1 R1	31.25 Time 1 R2	31.25 Time 2 R1	31.25 Time 2 R2	15.625 Time 1 R1	15.625 Time 1 R2	15.625 Time 2 R1	15.625 Time 2 R2	7.8125 Time 1 R1	7.8125 Time 1 R2	7.8125 Time 2 R1	7.8125 Time 2 R2	3.90625 Time 1 R1	3.90625 Time 1 R2	3.90625 Time 2 R1	3.90625 Time 2 R2
12	*Peltophorum pterocarpum* (DC.) Backer. ex K.Heyne.	นนทรี	bark	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
13	*Piper aurantuacum*	ชะพลู	leaf	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
14	*Piper betle* L.	พลู	leaf	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
15	*Piper chaba* Vahl.	ดีปลี	fruit	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
16	*Psidium guajava* L.	ฝรั่ง	leaf	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
17	*Punica granatum* L.	ทับทิม	skin	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
18	*Rhizophora mucronata* Poir	โกงกาง	bark	Et	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
19	*Sandoricum koetjape* (Burm. f.) Merr.	กระท้อน	root	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
20	*Terminalia bellerica* (Gaertn.) Roxb.	สมอภิเภก	fruit	Et	1+	1+	1+	1+	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
21	*Terminalia chebula* Retz.	สมอไทย	fruit	Et	2+	2+	2+	2+	3+	3+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+

R1 = replicate 1, R2 = replicate 2

Et = ethanol, Mt = methanol, Ace = acetone

(Upcroft & Upcroft, 2001)   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 parasite 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)

Appendix C
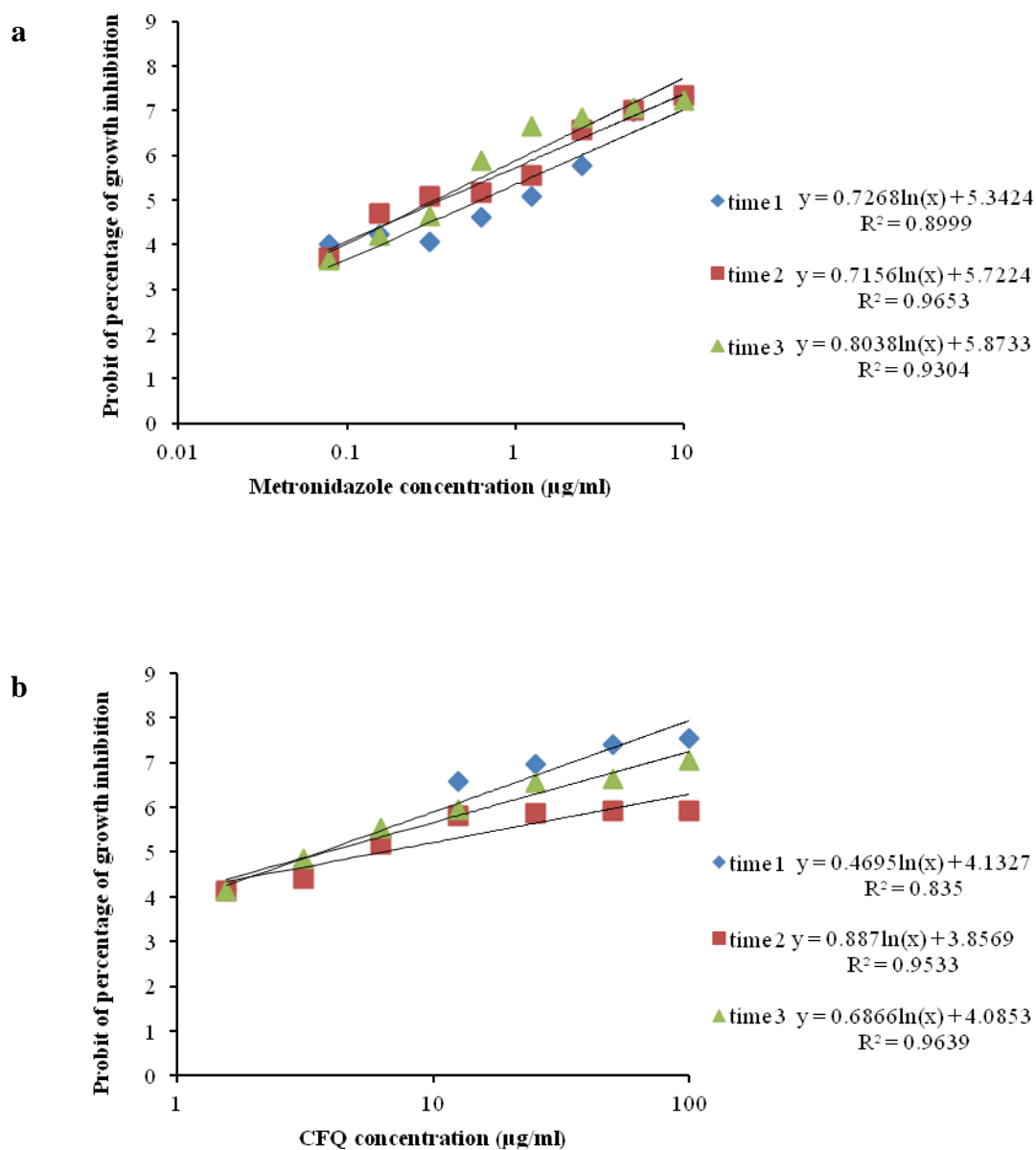
Figure 37 The probit values of metronidazole (a) and 1-hydroxy-2-hydroxymethylanthraquinone (b) against *E. histolytica* trophozoites after treated for 24 h of incubation period.

a

b
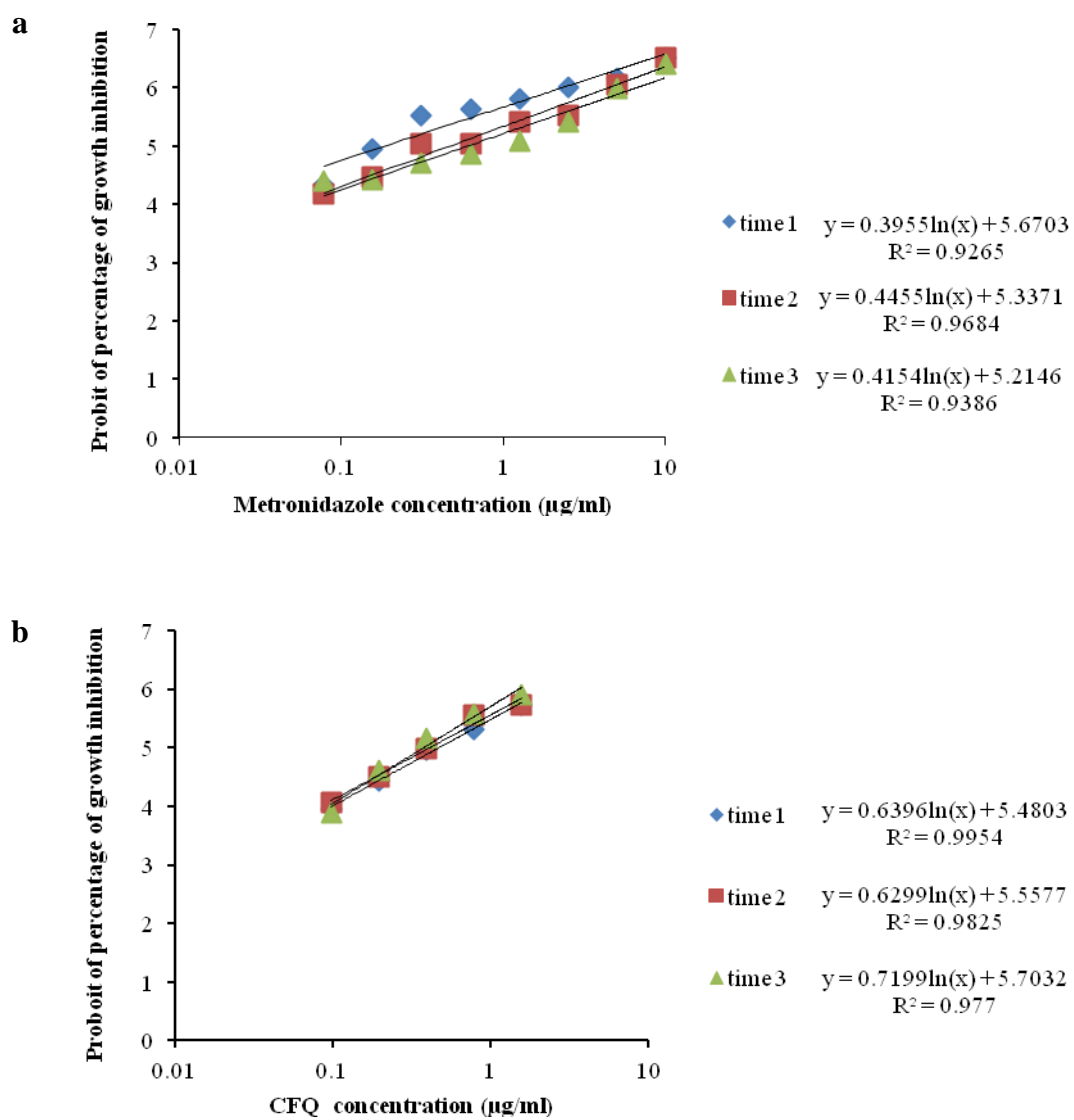
Figure 35 The probit values of metronidazole (a) and 1-hydroxy-2-hydroxymethylanthraquinone (b) against *G. intestinalis* trophozoites after treated for 24 h of incubation period.