In vitro Efficacy of Metronidazole and Secnidazole combination against clinical isolates of *E. histolytica*

A Dissertation submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Masters of Pharmacy.

Submitted by

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Submitted to

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Submission Date: 23rd January, 2018.

Declaration by the Research Candidate

I, Sabrina Akhter Mim, hereby declare that this dissertation, entitled "**In vitro Efficacy of Metronidazole and Secnidazole combination against clinical isolates of *E. histolytica*"** submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Masters of Pharmacy, is a genuine & authentic research work carried out by me under the guidance of Professor Dr. Sufia Islam, Department of Pharmacy, East West University, Dhaka. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

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Certificate by the Supervisor

This is to certify that the desertion, entitled "**In vitro Efficacy of Metronidazole and Secnidazole combination against clinical isolates of *E. histolytica*"** is an authentic and genuine research work done, under my guidance and supervision by Sabrina Akhter Mim (ID: 2016-3-79-012), in partial fulfillment for the requirement of the degree of Masters of Pharmacy.

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Certificate by the Chairperson

This is to certify that the desertion, entitled "**In vitro Efficacy of Metronidazole and Secnidazole combination against clinical isolates of *E. histolytica*"** is a bonafide research work done by Sabrina Akhter Mim (ID: 2016-3-79-012), in partial fulfillment for the requirements of the Degree of Masters of Pharmacy.

Prof. Dr. Chowdhury Faiz Hossain

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Dedication

This Research Paper is dedicated to

My beloved parents

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Abstract

Entamoeba histolytica is the etiological agent of amoebic dysentery and amoebic liver abscess (ALA). Worldwide, 40–50 million symptomatic cases of amoebiasis occur annually and 70,000 to 100,000 deaths are due to this infection. In amoebiasis Metronidazole, a 5-nitroimidazole drug is the drug of choice for intestinal disease caused by *Entamoeba histolytica*. However, the random use of Metronidazole can result in increased minimum inhibitory concentration (MIC) against *Entamoeba* species. As a result treatment failure may occur when treated with antiamoebic drug. Therefore, the aim of the study is to determine the efficacy of combination of Metronidazole & Secnidazole against clinical isolates of *E.histolytica.* The clinical isolates of *E. histolytica* were treated individually with Metronidazole & Secnidazole at different concentrations (2.56, 1.28, 0.64, 0.32, 0.16, 0.08 μg/ml). Drug sensitivity assay of the samples was carried out by using microtiter plates containing 100μl of parasite suspension (1×10^6 parasites/ml). Plates were incubated at 37ºC. After 4 hours the viable parasites were observed under microscope using haemocytometer. Viable counts of the *E. histolytica* treated with different concentration of drugs were compared with the control. The viable parasites in Control were $7.7X10^5$ cell/ml and $5.7X10^5$ cell/ml for testing the Metronidazole and Secnidazole respectively. Viable cell counts of *E. histolytica* were $3X10^5$, $3.5X10^5$ & $4X10^5$ cell/ml when different concentrations of Metronidazole (2.56, 1.28 & 0.64 μg/ml) were used. Viable cell counts of *E. histolytica* were $2.5X10^5$, $3.9X10^5$ & $4.6X10^5$ cell/ml when different concentrations of Secnidazole (2.56, 1.28 & 0.64 μg/ml) were used. Cell inhibition was maximum at the highest concentration of individual treatment (Metronidazole and Secnidazole). The parasite inhibition was occurred in a dose dependent manner. Cell inhibition of parasite was $3X10^5$ cell/ml when treated with 2.56 μg/ml of combination drug (Metronidazole+Secnidazole). The results indicate that both Metronidazole & Secnidazole showed adequate efficacy individually against *E. histolytica.* However, Combination (Metronidazole+Secnidazole) of these drugs didn't show any synergestic effect against *E. histolytica.*

Key words: *Entamoeba histolytica;* amoebiasis; microtiter plates; haemocytometer; incubation.

CHAPTER 1
Introduction

1.1 Background

Amoebiasis is a disease caused by the parasite *Entamoeba histolytica*. Hippocrates who described a patient with fever and dysentery first recognized it as a deadly disease. With the application of a number of new molecular biology-based techniques, tremendous advances have been made in our knowledge of the diagnosis, natural history, and epidemiology of amoebiasis. On a global basis, amoebiasis affects approximately 50 million persons each year resulting in 100,000 deaths (Petri *et al*, 2003). Amoebiasis is also very common in Bangladesh. Developments came later in 1855 when it was suggested that the disease might have a parasitic origin. Finally, *E. histolytica* was identified from a stool sample in 1875 by Fredor Losch. *Entamoeba histolytica* is the etiological agent of amoebic dysentery. *E. histolytica* is transmitted via ingestion of the cystic form (infective stage) of the protozoa. Viable in the environment for weeks to months, cysts can be found in focally contaminated soil, fertilizer, or water or on the contaminated hands of food handlers (Walsh *et al*, 1986). Recently there was an study published which discovered a strain named *Entamoeba bangladeshi* which can be distinguished visually from other members of the *Entamoeba* genus and also a reason for amoebiasis (Royer *et al*, 2012). Throughout the world, amoebiasis is the second leading cause of death from a parasitic disease. Although it is the second leading cause of death from parasitic diseases, about 90% of the people exposed to *E. histolytica* are asymptomatic or report very mild symptoms (Tanyuskel &, William 2003).

1.2 Amoebiasis

Amoebiasis is a condition in which intestines (gut) becomes infected with the parasite *E. histolytica*. *Entamoeba* is a group of single-celled parasites (living things that live in, or on, other living organisms) that can infect both humans and some animals. Amoebiasis can present with no, mild, or severe symptoms. Symptoms may include abdominal pain, mild diarrhoea, bloody diarrhea or severe colitis with tissue death and perforation. Most people who become infected with *E. histolytica* do not develop any symptoms. However, symptoms may develop if the parasite causes inflammation of the lining of gut. In some people, *E. histolytica* can also get into the bloodstream from the gut and spread around the body to the liver, lungs and sometimes other organs. Amoebiasis remains an important health problem in tropical countries where sanitation infrastructure and health are often inadequate. Only about 10% to 20% of people who are

infected with *E. histolytica* become sick from the infection. It can affect anyone, although it is more common in people who live in tropical areas with poor sanitary conditions *(*Farrar *et al*, 2013). Amoebiasis is a common infection of the human gastro-intestinal tract. Amoebiasis is more closely related to poor sanitation and socioeconomic status than to climate. It has worldwide distribution. It is a major health problem in China, South East and West Asia and Latin America, especially Mexico and Bangladesh. In 1969, WHO defined amoebiasis, a condition in which a patient harbouring the organism *Entamoeba histolytica* in the bowel. Culture of *E. histolytica* is a long and laborious process. There are three basic types of culture systems of *E. histolytica*, xenic, in which the parasite is grown in the presence of an undefined flora; monoxenic, in which the parasite is grown in the presence of a single additional species; and axenic in which the parasite is grown in the absence of any other metabolizing cells (Weedall & Hall, 2011).

1.3 Amoeba

Amoeba often called amoeboid. It is a type of cell which has the ability to alter its shape. An amoeba is any of several tiny, one-celled protozoa in the phylum (or primary division of the *In vitro* efficacy study of metronidazole & secnidazole against clinical isolates of *E. histolytica and* animal kingdom) Sarcodina. The well-known type Amoeba Proteus lives in freshwater and salt water, in soil, and as parasites in moist body parts of animals. They are composed of cytoplasm (cellular fluid) divided into two parts: a thin, clear, gel-like outer layer that acts as a membrane (ectoplasm); and an inner, more watery grainy mass (endoplasm) containing structures called organelles. Amoebas may have one or more nuclei, depending upon the species. It is found on decaying bottom vegetation of freshwater streams and ponds (Visvesvara *et al*, 2007).

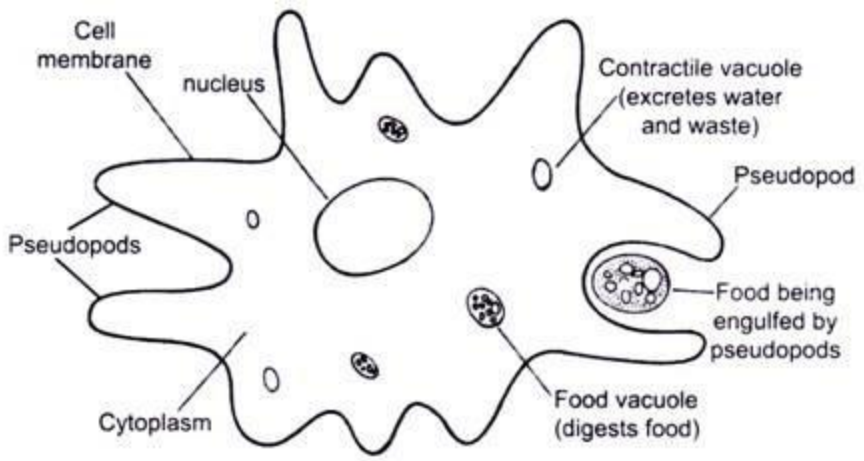

Fig. 9.7 *Amoeba proteus*

Figure 1. 1 : Amoeba

There are many varieties of free-living amoeba, but only four genera have been causally associated with disease in humans. These are *Acanthamoeba*s, *Balamuthia mandrillaris* (the only know species of *Balamuthia*), *Naegleriafowleri* (sometimes considered not to be an amoeba at all, but more closely related to *Leishmania*and *Trypanosoma*) and *Sappiniapedata*. They are distinct from the more famous *Entamoeba histolytica* (an obligate anaerobic parasite which can cause amoebiasis, amoebic dysentery and amoebic liver abscesses). *Acanthamoeba*s and *B. mandrillaris* are opportunistic pathogens causing infections of the CNS, lungs, sinuses and skin, mostly in immunocompromised humans. *B. mandrillaris* is also associated with disease in immunocompetent children, and *Acanthamoeba*s pp. cause a sight-threatening keratitis, mostly in contact lens wearers. *N. fowleri* causes an acute and fulminating meningoencephalitis in immunocompetent children and young adults. A few human cases of encephalitis caused by *Sappiniadiploidea* have been described (Petri and Tanyuskel, 2003).

1.4 Distribution

Acanthamoebas pp. is found in soil, dust, air and water (eg, swimming pool, domestic and sewage), ventilation and air conditioning systems. They have been isolated in hospitals, medicinal pools, dental treatment units, dialysis machines and contact lenses. They have also been found in mammalian cell cultures, human nostrils and throats and human and animal brain,

skin, and lung tissues. In cell cultures they are commonly contaminants. This is how they were discovered in the 1950s - they grew on cell cultures grown for the polio vaccine. Acanthamoebas pp. can also be found in fish and have been isolated from the nasal and throat mucosa of healthy humans. B. mandrillarishas not been isolated from the environment but has been isolated from autopsy specimens of infected humans and animals. N. fowleri is also ubiquitous and found in soil and warm fresh water. Sappinias pp. is found in soil and tree bark. Both Acanthamoebas pp. and B. mandrillariscan act as hostsm for other bacterial infections - eg, legionellosis (Shadrach *et al*, 2005).

1.5 Life cycle of Amoeba

Reproduction in amoeba is a periodic process taking place at intervals. Reproduction in amoeba chiefly occurs by asexual method, i.e., by binary fission, multiple fission and sporulation.

(i) Binary fission

It is the most common mode of reproduction. In this process, the whole body divides into two daughter amoebae by mitosis. The division involves nuclear division (karyokinesis) followed by division of cytoplasm (cytokinesis) (fig. 1.2). This takes place during favourable conditions.

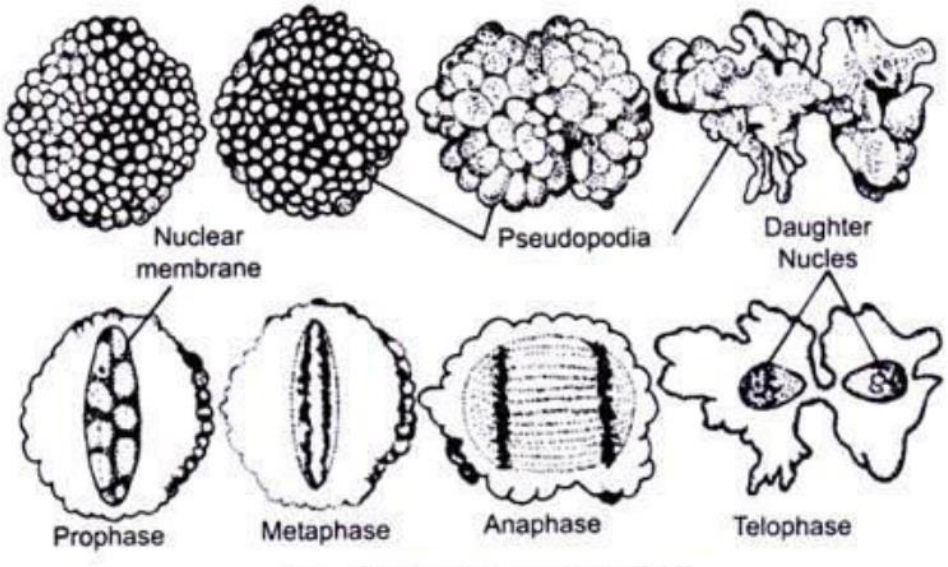

Figure 1. 2 : Binary fission in Amoeba

(ii) Sporulation

Under un-favourable conditions amoeba reproduces by formation of spores internally. It starts with the breakdown of nuclear membrane and release of chromatin blocks into the cytoplasm. Each chromatin blocks acquires a nuclear membrane and becomes a small daughter nuclei. The newly formed nuclei get surrounded by cytoplasm to form amoebulae. The peripheral cytoplasmic layer of amoebulae forms a tough and resistant sporemembrane or spore case (fig. 1.3). About 200 such spores are formed inside a single parent amoeba. Finally the body of parent amoeba disintegrates to release the spores. The spore remain inactive for some time and on getting favourable conditions each spore forms a young amoeba.

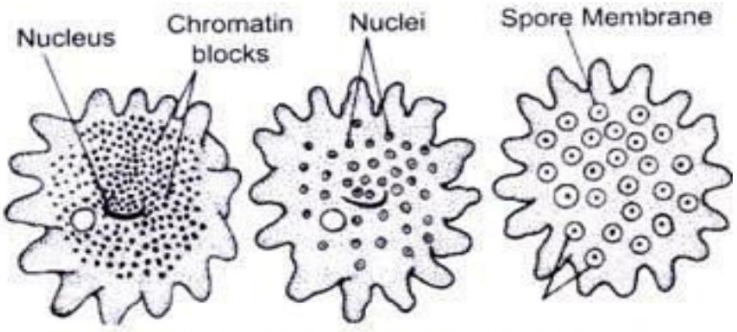

Figure 1. 3 : Sporulation in Amoeba

(iii) Multiple fission

In un-favourable conditions, amoeba divides by multiple fission. It withdraws its pseudopodia, becomes spherical and secretes three layered cyst around itself. Its nucleus undergoes repeated mitosis division forming 500- 600 daughter nuclei. Each daughter nucei gets surrounded by mass of cytoplasm and divides into minute amoebulae. On getting favourable conditions the cyst ruptures to release the amoebulae which soon grows into adult amoeba.

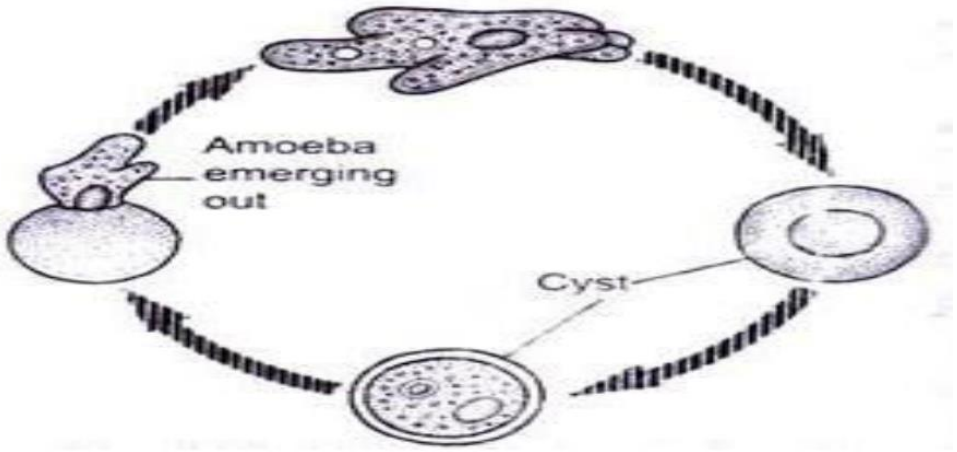

Figure 1. 4 : Multiple fission in Amoeba

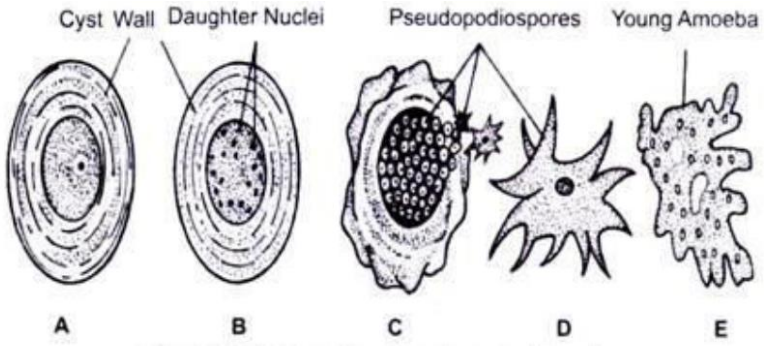

Figure 1. 5 : Stages of multiple fission in Amoeba

1.6 Regeneration

Amoeba has tremendous power of regeneration. If it is cut into small pieces, each piece regenerates into a new amoeba, however, a piece without nuclear fragment does not regenerate (Medhekar 2016).

1.7 Amoebiasis

Amoebiasis is an infection of intestine caused by a parasite *Entamoeba histolytica*. *It* remains an important health problem in tropical countries where sanitation infrastructure and health are often inadequate. This disease can present with no, mild, or severe symptoms. Only about 10% to 20% of people who are infected with *Entamoeba histolytica* become sick from the infection. It can affect anyone, although it is more common in people who live in tropical areas with poor

sanitary conditions. Amoebiasis is a common infection of the human gastro-intestinal tract. Amoebiasis is more closely related to poor sanitation and socioeconomic status than to climate. It has worldwide distribution. It is a major health problem in China, South East and West Asia and Latin America, especially Mexico and Bangladesh. In 1969, WHO defined amoebiasis, a condition in which a patient harbouring the organism *Entamoeba histolytica* in the bowel. Culture of *Entamoeba histolytica* is a long and laborious process. There are three basic types of culture systems of *Entamoeba histolytica*: xenic, in which the parasite is grown in the presence of an undefined flora; monoxenic, in which the parasite is grown in the presence of a single additional species; and axenic in which the parasite is grown in the absence of any other metabolizing cells (Farrar *et al*, 2013).

1.8 Etiology

Amebiasis is a parasitic infection caused by the protozoal organism *E. histolytica*, which can give rise both to intestinal disease (eg, colitis) and to various extraintestinal manifestations, including liver abscess (most common) and pleuropulmonary, cardiac, and cerebral issemination. The genus Entamoeba contains many species, some of which (ie, *E. histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba polecki*, *Entamoeba coli*,and *Entamoeba hartmanni*) can reside in the human interstitial lumen. Of these, *E. histolytica* is the only one definitely associated with disease; the others are considered no pathogenic. Studies have recovered *E. dispar* and *E. moshkovskii* from patients with gastrointestinal (GI) symptoms, but whether these species cause these symptoms remains to be determined. Although *E. dispar* and *E. histolytica* cannot be differentiated by means of direct examination, molecular techniques have demonstrated that they are indeed 2 different species, with *E. dispar* being commensal (as in patients with HIV infection) and E histolytica pathogenic. It is currently believed that many individuals with Entamoeba infections are actually colonized with *E. dispar*, which appears to be 10 times more common than E histolytica; however, in certain regions (eg, Brazil and Egypt), asymptomatic *E. dispar* and *E. histolytica* infections are equally prevalent. In Western countries, approximately 20%-30% of men who have sex with men are colonized with *E. dispar*. *E histolytica* is transmitted primarily through the fecal-oral route. Infective cysts can be found in fecally contaminated food and water supplies and contaminated hands of food handlers. Sexual transmission is possible, especially in the setting of oral-anal practices (anilingus). Poor

nutrition, through its effect on immunity, has been found to be a risk factor for amebiasis (Dhawan 2017).

1.9 Entamoeba histolytica

Entamoeba histolytica is a pseudopod forming, anaerobic parasitic amoebozoa and a part of the genus Entamoeba. Predominantly infecting humans and other primates causing amoebiasis, *Entamoeba histolytica* is estimated to infect about 50 million people worldwide (Weedall and Hall, 2011).

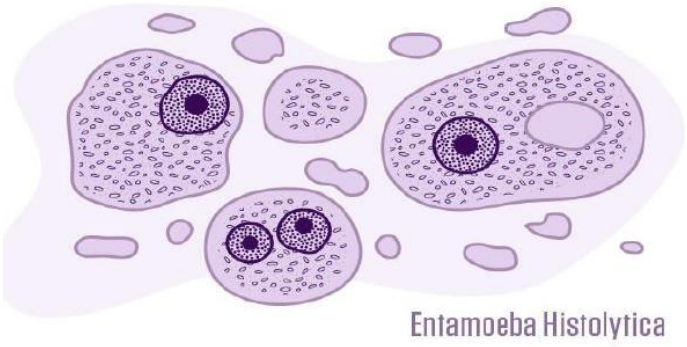

Figure 1. 6 : Microscopic image of Entamoeba histolytica

Entamoeba histolytica, is the etiological agent of amoebic dysentery and amoebic liver abscess (ALA). Worldwide, 40–50 million symptomatic cases of amoebiasis occur annually and 70,000 to 100,000 deaths due to this infection. *Entamoeba histolytica*, associated with high morbidity and mortality continues to be a major public health problem throughout the world. Asymptomatic individuals account for almost 90 per cent of the infections. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favour transmission and increased disease burden. Prevalence varies from country to country and within a country. *Entamoeba histolytica* infections can be detected through fecal microscopy, culture, PCR, and antigen detection (Ryan & Ray, 2004).

1.10 Scientific classification

Kingdom Protista

Subkingdom Protozoa

Phylum Sarcomastigophora

Class Lobosea

Order Amoebida

Family Entamoebida

Genus+Species *Entamoeba histolytica*

1.11 Life cycle

Entamoeba histolytica is a monogenetic parasite as its life cycle is completed in a single host i.e., man. Three distinct morphological forms exist in its life cycle. - Trophozoite, Pre-cystic stage and Cystic stage (Saritha, 2015).

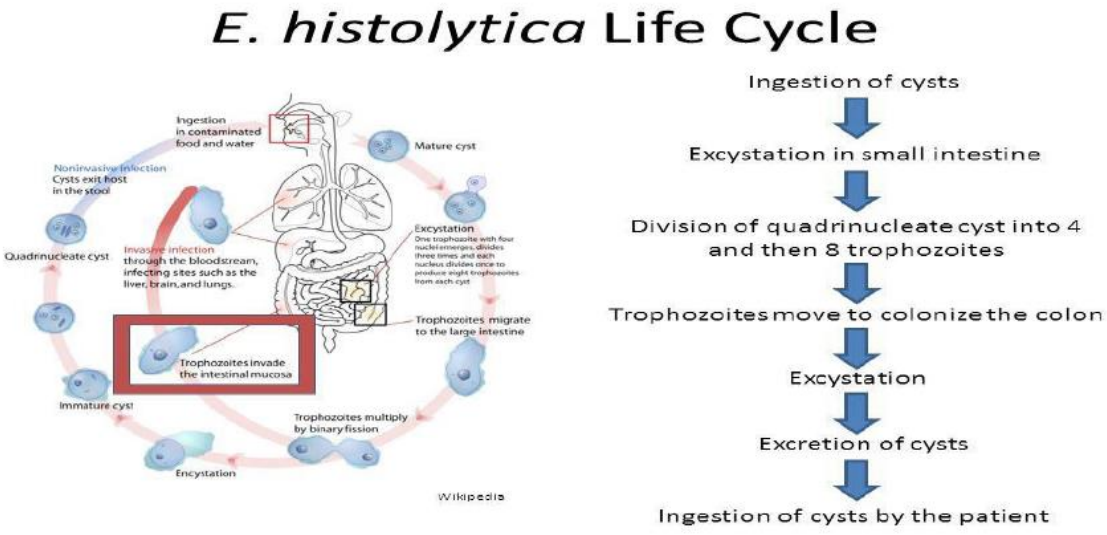

Figure 1. 7 : Life cycle of E. histolytica

1.12 Transmission

A matured quadrinucleate cyst of *Entamoeba histolytica* is the infective stage of the parasite. Transmission of *Entamoeba histolytica* from one person to another occurs due to ingestion of these cysts. Fecal contamination of edible substances and drinking water are the primary cause of infection. Following are the mode of transmission of this parasite.

(a) Fecal-oral route:

In majority of cases infection takes place through intake of contaminated uncooked vegetables and fruits. Insect vectors like flies, cockroaches and rodents act as agent to carry infective cysts to the food and drink. Sometimes drinking water supply contaminated with infected faces give rise to epidemics.

(b) Oral-rectal contact:

Sexual transmission by oral-rectal contact is also one of the modes of transmission, specially among male homosexuals (Sodeman, 1996).

1.13 Structure

The life cycle of *Entamoeba histolytica*, includes three stages,

1. Trophozoite stage,
2. Precystic stage and
3. Cystic stage.

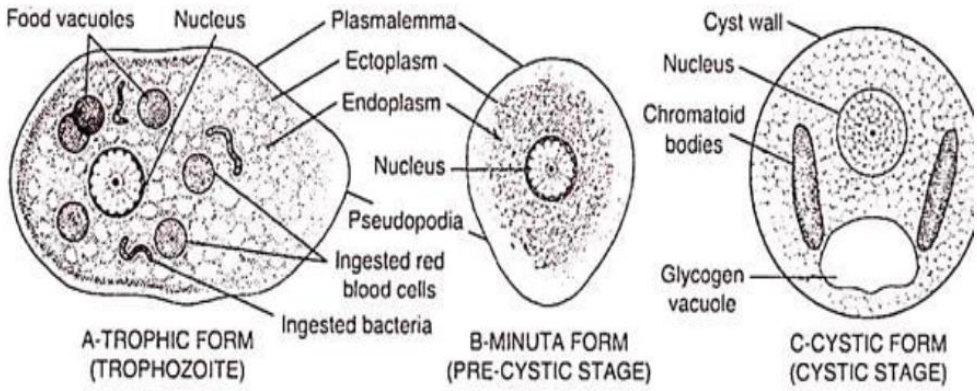

Figure 1. 8 : Stages of Entamoeba histolytica

1.13.1 Trophozoite stage

Trophozoite is the motile, feeding and pathogenic stage of *Entamoeba histolytica*. It measures about 20-30 microns in diameter. Trophozoite is surrounded by the limiting membrane called plamalemma.

1.13.2. Precystic stage

Some of the daughter amoebae that are entered into the lumen of intestine develop transform into precystic stage. It is the nonmotile, nonfeeding and nonpathogenic stage of *Entamoeba histolytica*. It is relatively smaller in size, measuring about 10-20 micrometers.

1.13.3. Cystic stage

It is found in the lumen of the large intestine. It is round in shape and is surrounded by a thin, delicate and highly resistant wall. The precess of development of cyst wall is called encystation which is a means to tide over the unfavourable conditions that the parasite is going to encounter while that the parasite is going to encounter while passing to a new host (Sodeman, 1996).

1.14 Pathology

In the vast majority of cases, infection is asymptomatic and the carrier is unaware they are infected. However, in an estimated 10% of cases *E. histolytica* causes disease. Once the trophozoites are excysted they colonize the large bowel, remaining on the surface of the mucus layer and feeding on bacteria and food particles. Occasionally, and in response to unknown stimuli, trophozoites move through the mucus layer where they come in contact with the epithelial cell layer and start the pathological process. *E. histolytica* has a lectin that binds to galactose and N-acetylgalactosamine sugars on the surface of the epithelial cells, The lectin normally is used to bind bacteria for ingestion. The parasite has several enzymes such as pore forming proteins, lipases, and cysteine proteases, which are normally used to digest bacteria in food vacuoles but which can cause lysis of the epithelial cells by inducing cellular necrosis and apoptosis when the trophozoite comes in contact with them and binds via the lectin. Enzymes released allow penetration into intestinal wall and blood vessels, sometimes on to liver and other organs. The trophozoites will then ingest these dead cells. This damage to the epithelial cell layer attracts human immune cells and these in turn can be lysed by the trophozoite, which releases the immune cell's own lytic enzymes into the surrounding tissue, creating a type of chain reaction and leading to tissue destruction. This destruction manifests itself in the form of an 'ulcer' in the tissue, typically described as flask-shaped because of its appearance in transverse section. This tissue destruction can also involve blood vessels leading to bloody diarrhea, amebic dysentery. Occasionally, trophozoites enter the bloodstream where they are transported typically to the liver via the portal system. In the liver a similar pathological sequence ensues, leading to amebic liver

abscesses. The trophozoites can also end up in other organs, sometimes via the bloodstream, sometimes via liver abscess rupture or fistulas. In all locations, similar pathology can occur (*Anderson, 2017)*

1.15 Entamoeba bangladeshi

In 2010–2011, during analysis of feces positive for Entamoeba organisms by microscopy or culture but negative for *Entamoeba histolytica*, *E. dispar*, and *E. moshkovskii* by PCR, a new species was identified, which was named *Entamoeba bangladeshi* nov. sp. in recognition of the support of the Bangladesh community for this research. By light microscopy, we detected no apparent differences between *Entamoeba bangladeshi* and *Entamoeba histolytica*. The physical resemblance between *Entamoeba histolytica* and *Entamoeba bangladeshi* is notable because direct microscopic examination of fecal samples is still used as a diagnostic tool in areas to which these species are endemic to detect *Entamoeba histolytica* parasites. (Haque & Mondal, 2009). To further characterize *Entamoeba bangladeshi*, it has the ability to established in xenic culture, and it displayed the ability to grow at 37°C and 25°C, a characteristic shared with *E. moshkovskii* and *E. ecuadoriensis* but that distinguishes it from *Entamoeba histolytica* and *E. dispar*. Tests negative in *Entamoeba histolytica* ELISA and in species-specific PCRs. Currently only identifiable by its small subunit ribosomal RNA gene sequence (Stensvold CR, 2011).

1.16 Antiamoebic drugs

Infection is primarily treated by instituting antiamoebic therapy. The detection of *Entamoeba histolytica* and *Entamoeba bangladeshi* is an important goal of the clinical microbiology laboratory. This is because amebiasis is presently one of the three most common causes of death from parasitic disease. Drugs of choice for invasive amoebiasis are tissue active agents, like metronidazole, Ornidazole and chloroquine or the more toxic emetine derivatives, including dehydroemetine. Metronidazole is derived from 5- nitroimdazole which kill the trophozoites by alterations in the protoplasmic organelles of the amoeba. Nitroimidazole therapy leads to clinical response in approximately 90% of patients with mild-to-moderate amebic colitis. Because intraluminal parasites are not affected by nitroimidazoles, nitroimidazole therapy for amebic colitis should be followed by treatment with a luminal agent (eg, paromomycin or diloxanide

furoate) to prevent a relapse. Amebic liver abscess of up to 10 cm can be cured with metronidazole without drainage. Clinical defervescence should occur during the first 3-4 days of treatment. Failure of metronidazole therapy may be an indication for surgical intervention. Chloroquine has also been used for patients with hepatic amebiasis. Dehydroemetine (available from the Centers for Disease Control and Prevention [CDC] has been successfully used but, because of its potential myocardial toxicity, is not preferred). Broad-spectrum antibiotics may be added to treat bacterial super infection in cases of amoebic colitis and suspected perforation. Bacterial co-infection of amebic liver abscess has occasionally been observed (both before and as a complication of drainage), and adding antibiotics to the treatment regimen is reasonable in the absence of a prompt response to nitroimidazole therapy (Stanley, 2003).

1.17 Metronidazole

Metronidazole is an antibiotic that is used to treat a wide variety of infections which marketed under the brand name Flagyl among others. It works by stopping the growth of certain bacteria and parasites (Brayfield, 2014). Metronidazole (1-(2-hydroxyethyl)-2- methyl-5-nitroimidazole) is the drug of choice for the treatment of infections caused by anaerobic or microaerophilic microorganisms and has been used in clinical practice for >25 years. The molar mass of Metronidazole is 171.15 g/mol. Metronidazole began to be commercially used in 1960 in France. It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system (WHO 2015). It is effective for giardiasis, trichomoniasis, and amebiasis. It is the drug of choice for a first episode of mild-to-moderate Clostridium difficile colitis. Metronidazole is available by mouth, as a cream, and intravenously (Cohen, 2010)

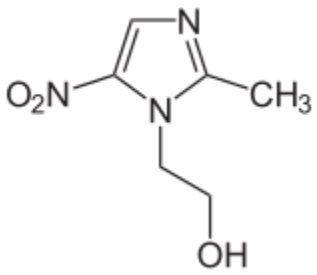

Figure 1. 9 : Chemical and 3D structure of Metronidazole

For the treatment of anaerobic infections and mixed infections, surgical prophylaxis requiring anaerobic coverage, Clostridium difficile-associated diarrhea and colitis, Helicobacter pylori infection and duodenal ulcer disease, bacterial vaginosis, Giardia lamblia gastro-enteritis, amebiasis caused by *E. histolytica* , acne rosacea (topical treatment), and Trichomonas infections (Freeman *et al*, 1997).

1.18 Pharmacokinetics of Metronidazole

Metronidazole appears in cerebrospinal fluid, saliva, and breast milk in concentrations similar to those found in plasma. Less than 20% is protein bound. It is absorbed rapidly and completely if also given through orally. It is distributed very well into mucosa, saliva, bone and CSF. It is metabolized in liver. Routes of elimination are via urine (60% to 80%) and feces (6% to 15%). Renal Clearance is approximately 10 mL/min per 1.73 m2. The plasma ½ life of metronidazole is about 8 hours (Thomson, 1985).

1.19 Metabolism of Metronidazole

Approximately 30-60% of an oral or IV dose of metronidazole is metabolized in the liver by hydroxylation, side-chain oxidation, and glucuronide conjugation. The major metabolite, 2-hydroxy metronidazole, has some antibacterial and antiprotozoal activity. Four other nitro-group-containing metabolites have been identified, each derived from side-chain oxidation of ethyl and/or methyl group. They include 1- acetic acid-2-methyl-5-nitroimidazole and 1-(2-hydroxyethyl)-2-carboxylic acid-5- nitroimidazole salt. The liver is the main site of metablism, and this accounts for over 50% of the systemic clearance of Metronidazole. The 2 principal metabolites result from oxidation of side chains, a hydroxy derivative and an acid. The hydroxy metabolite has a longer half-life (about 12 hr) and nearly 50% of the antitrichomonal activity of Metronidazole. Formation of glucuronides also is observed. Small quantities of reduced mebolites, including ring-cleavage products, are formed by the gut flora. The urine of some patients may be reddish-brown owing to the presence of unidentified pigments derived from the drug (Hardman *et al,* 2001).

1.20 Mechanism of action of Metronidazole

Metronidazole is of the nitroimidazole class. It inhibits nucleic acid synthesis by disrupting the DNA of microbial cells (Eisenstein, 2007). Metronidazole can enter the cell through passive diffusion, where a nitro group is subsequently reduced to reactive cytotoxic nitro radicals by reduced ferredoxin or flavodoxin. Ferredoxin and flavodoxin function as electron acceptors of pyruvate. ferredoxin oxidoreductase, hydrogenase, and some other enzymes found specifically in microaerophilic bacteria and protozoan parasites. Under aerobic conditions, the nitro radicals can be oxidized by oxygen, which leads to futile cycling and detoxification of the drug. During this reaction, superoxide radical anions are formed that may also be toxic for the anaerobic organisms (Thomson, 1985). Metronidazole is a prodrug. Unionized Metronidazole is selective for anaerobic bacteria due to their ability to intracellularly reduced Metronidazole to its active form. This reduced Metronidazole then covalently binds to DNA, disrupt its helical structure, inhibiting bacterial nucleic acid synthesis and resulting in bacterial cell death. Treatment with Metronidazole is usually very effective (Brayfield, 2014).

1.21 Secnidazole

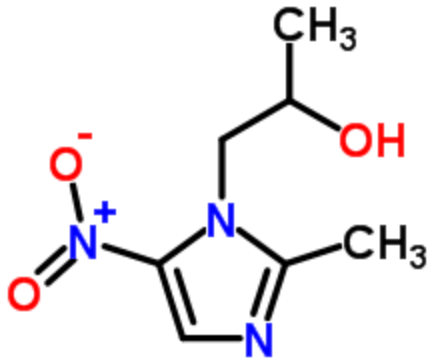

Figure 1. 10 Chemical and 3D structure of Secnidazole

Secnidazole is structurally related to the commonly used 5-nitroimidazoles, Metronidazole and Tinidazole. These drugs share a common spectrum of activity against anaerobic micro-organisms and they appear particularly effective in the treatment of amoebiasis, giardiasis, trichomoniasis

and bacterial vaginosis. Secnidazole is rapidly and completely absorbed after oral administration and has a longer terminal elimination half-life (approximately 17 to 29 hours) than commonly used drugs in this class. in patients with intestinal amoebiasis or giardiasis, clinical or parasistological cure rates of 80 to 100% are achieved after treatment with a single dose of secnidazole 2g (30 mg/kg in children), similar to the response rates achieved with multiple dosage regimens of metronidazole or tinidazole. Patients with hepatic amoebiasis appears to respond well to 5- to 7-day therapy with secnidazole, but the efficacy of this drug regimen requires further evaluation in larger numbers of patients. After administration of a single dose of secnidazole, parasitological eradication was achieved in approximately 92 to 100% of patients with urogenital trichomoniasis. Patients with bacteria vaginosis respond at least as well to a single dose of Secnidazole as to single-dose Tinidazole, or single- or 7-day treatment with Metronidazole; clinical improvement and/or microbiological evidence of cure was attained in approximately 59 to 96% of patients. In the clinical trials reviewed, Secnidazole was well tolerated; most adverse events were gastrointestinal in nature and did not require treatment intervention or withdrawal from therapy. In summary, available evidence suggests that Secnidazole is as efficacious as other 5-nitroimidazole drugs in the treatment of protozoal infections and bacterial vaginosis. The convenience and ease of administration associated with single-dose therapy, combined with a good tolerability profile, make Secnidazole a suitable option to other single-dose treatments and an attractive alternative to multiple dosage regimens with other drugs in this class (Gillis & Wiseman, 1996)

CHAPTER 2
Literature review

2.1 In vitro Sensitivity of Different Brands of Antiamoebic Drugs (Metronidazole Tablets) Against Clinical Isolates of *Entamoeba histolytica* in Bangladesh

A study was conducted with EH treated with different brands of Metronidazole from Bangladeshi pharmaceutical industries. Twelve different brands of Metronidazole tablets were used where some tablets were from large Parma industries and some from small pharmaceuticals according to their business. Parasite count was adjusted to 3x106 parasites mL-1 in a medium. *In vitro* drug sensitivity assay of the samples was carried out by using 96 wells microtiter plates after treatment with different concentrations of Metronidazole. No statistical significance was observed in terms of viable parasites with the Metronidazole tablets from three big pharmaceuticals at the concentration of 2.3, 3.5 and 4.6 μM when compared with the standard metronidazole. (Sarker *et al*, 2008).

2.2 *In vitro* activity of antiamoebic drugs against clinical isolates of *Entamoeba histolytica* and *Entamoeba dispar*

This study was aimed to assess the *In vitro* susceptibility of clinical isolates of *E. histolytica* and *E. dispar* to Metronidazole, Chloroquine, Emetine and Tinidazole. A total of 45 clinical isolates (15 *E. histolytica* and 30 *E. dispar*) were maintained in polyxenic cultures followed by monoxenic cultures. *In vitro* drug sensitivity (IC50) of clinical isolates and standard reference strain of *E. histolytica* (HM1: IMSS) was assessed by nitro blue tetrazolium (NBT) reduction assay after exposure to various concentrations of each drug. The results showed that all clinical isolates had a higher IC50 compared to reference strain to all the four drugs. *E. histolytica* isolates appeared to be more susceptible compared to *E. dispar* isolates and the reference strain of *E. histolytica* after treatment with Metronidazole, Chloroquine, Emetine and Tinidazole (Bansal *et al*, 2004).

2.3 Study of Combination Regimens of Anti-Amoebic Drugs for the Treatment of Amoebic Dysentery Caused by *E. histolytica*

Amoebiasis is one of the most common health problems in the developing countries. Poverty, ignorance, overcrowding, poor sanitation and malnutrition favor transmission and increase disease burden. The objective of the study was to determine the sensitivity of the combination

regimens of anti-amoebic drugs against clinical isolates of *E. histolytica*. The clinical isolates of *E. histolytica* were treated with Metronidazole, Ornidazole, (Mtronidazole+Ornidazole), Secnidazole, (Metronidazole+Secnidazole), Tinidazole, and (Metronidazole+Tinidazole) at different concentrations (12, 6, 3 &1.5 mg/ml). Result showed that combination of metronidazole and ornidazole (1.5 mg/ml) inhibit the growth of E histolytica and it has found significantly different when compared with the control ($p<0.05$). Combination of Tinidazole and Metronidazole at the concentration of 6 and 12mg/ml has also found statistically significant ($p<0.05$) to inhibit the growth of *E. histolytica* when compared with the control. In this study it was shown that treatment with combination drugs may be a useful alternative to inhibit the growth of *E. histolytica* (Suki, 2015; Unpublished).

2.4 *Entamoeba bangladeshi* nov. sp., Bangladesh

In this survey both diarrheal and surveillance specimens of faeces were collected from a cohort of children living in Mirpur, Dhaka, Bangladesh for microscopical examination of the parasite present in the specimens. A total of 2,039 samples were examined microscopically (0.9% saline smear) and/or by fecal culture for amebic trophozoites and cysts. The study samples were collected from 2010 to 2011. A new species was identified which was positive for *Entamoeba* organisms by microscopy or culture. However, the species was negative for *E. histolytica, E. dispar,* and *E. moshkovskii* by PCR. This new spesies is named *Entamoeba bangladeshi* nov. sp. In recognition of the support of the Bangladesh community for this research (Royer *et al*, 2012).

2.5 Efficacy of macrolides vs. metronidazole against Entamoeba histolytica clinical isolates.

This study has shown that current treatment of Entamoeba histolytica infection requires the use of several agents that are effective at different sites of the body. Commonly administered agents such as nitroimidazoles have a high rate of gastrointestinal side effects and their use is restricted during pregnancy. In order to offer new choices, four macrolide antibiotics (Erythromycin, Clarithromycin, Azithromycin, Josamycin) and Metronidazole were tested for their in vitro activity against E. histolytica. Ten clinically isolated strains from an endemic area (Santo Domingo, Dominican Republic) were tested after polyxenical culture. Protozoan viability was significantly reduced by josamycin after 24 and 48 hours of incubation at a concentration of > or

= 50 mg/l, which was slightly higher than that of metronidazole (25 mg/l). No resistance to Metronidazole was found. The antiamebic activity of Azithromycin, Clarithromycin and Erythromycin was significant at drug concentrations > or = 100 mg/l. High doses of Josamycin, which is a very well tolerated drug, may serve as a useful therapeutic agent in the presence of E. histolytica infection. (Georgopoulos *et al*, 2001)

CHAPTER 3
Objective of the Study

Objective of the Study

The objectives of the study are-

1. To investigate the efficacy of Metronidazole and secnidazole against *E.histolytica* at different concentrations.
2. To investigate the efficacy of combination of both Metronidazole & Secnidazole on *E.histolytica* at different concentrations.

CHAPTER 4
Materials and Methods

4.1 Clinical isolation of cell

Clinical isolates of *E. histolytica* and *E. bangladeshi* were harvested from 24 hours old cultures and suspended in a LYI-S-2 medium. Axenic medium (LYI-S-2) consists of liver digest, yeast extract, iron, serum. The parasite count was adjusted to 1×10^6 parasites/ml in medium by haemocytometer. Isolation is usually achieved by growing the species in an environment that was previously sterilized, and was thereby rid of contaminating organisms. This isolation was performed in the Parasitology Laboratory, Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

4.2 Sample collection:

The drug metronidazole and secnidazole used in the study was procured as pure salt from INCEPTA Pharmaceutical Industry, which is one of the leading companies of Bangladesh. The sample was properly checked for their physical appearance, stability, batch number, manufacturing date, expiry date, manufacturing license number, D.A.R. number. The parasites were collected from the research department of ICDDR'B in Mohakhali, Dhaka. This investigation was performed in the parasitology Laboratory of ICDDR'B during the time of September 2017 to January 2018.

4.3 Preparation of antimicrobial drug:

1. 2.56 µg of metronidazole was weighted carefully by using a calibrated analytical balance.

3. The calculated amount of sample was dissolved in 1ml distilled water.

4. The solution was mixed by shaking carefully.

5. Then The stock solution was stored in refrigerator.

4.4 Apparatus and Instruments:

1) Eppendrof
2) Falcone tube
3) Micropipettes
4) Microtips

5) Beaker

6) Microtiter plate

7) Analytical balance

8) Vortex machine

9) Haemocytometer

10) Microscope

11) Incubator

12) Autoclave

13) Laminar flow

14) Trypan blue reagent

4.4.1 Incubation

An incubator is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as the carbon dioxide (CO2) and oxygen content of the atmosphere inside. Incubators are essential for a lot of experimental work in cell biology, microbiology and molecular biology and are used to culture both bacterial as well as eukaryotic cells.

Figure 4. 1 : Incubator

4.4.2 Microscope

A microscope (from the Ancient Greek: "small" and "to look" or "see") is an instrument used to see objects that are too small to be seen by the naked eye. Microscopy is the science of investigating small objects and structures using such an instrument. Microscopic means invisible to the eye unless aided by a microscope.

Figure 4. 2 : Microscope

4.4.3 Haemocytometer

The haemocytometer is a device usually used and originally designed to count blood cells or parasite cells. The haemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. It is therefore possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.

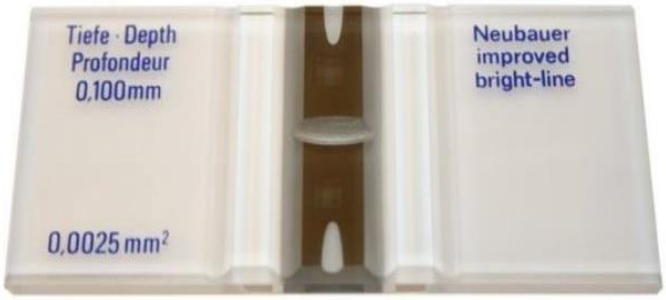

Figure 4. 3 : Haemocytometer

4.4.5 Autoclave

An **autoclave** is a pressure chamber used to carry out industrial processes requiring elevated temperature and pressure different from ambient air pressure. Autoclaves are used in medical applications to perform sterilization. Many autoclaves are used to sterilize equipment and supplies by subjecting them to high-pressure saturated steam at 121 °C (249 °F) for around 15–

20 minutes depending on the size of the load and the contents. The autoclave was invented by Charles Chamberland in 1879.

Figure 4. 4 : Autoclave

4.4.6 Microtiter plate:

A microtiter plate (spelled Microtiter is a registered trade name in the United States) or microplate or microwell plate or multiwell, is a flat plate with multiple "wells" used as small test tubes. The microplate has become a standard tool in analytical research and clinical diagnostic testing laboratories.

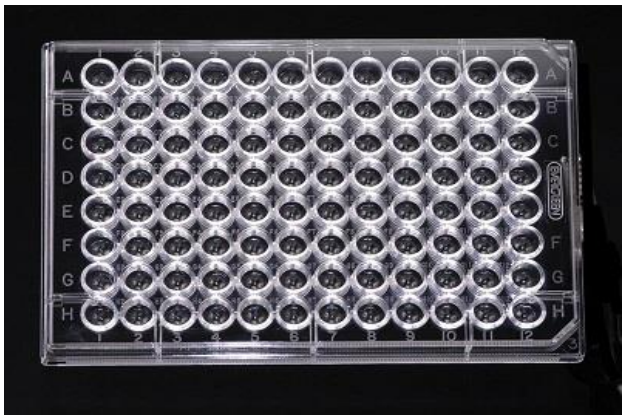

Figure 4. 5 : Microtiter plate

4.4.7 In vitro drug sensitivity assay procedure

1. Drug sensitivity assay of the samples was carried out by using microtiter plates. In wells (A-1) to (F-1), 100 µl medium was added except (B-1) and (C-1)
2. Then in (C-1), 100 µl metronidazole stock solution was added and serial dilutions of the drugs were performed down {(C-1) to (F-1)}.

3. Then the plate was mixed properly.
4. After that 100 µl of the medium from the well (F-1) was discarded to maintain the equality of the concentration of the drugs.
5. Further 100 µl of parasite suspension (1×10^6 parasites/ml) was added to all the wells {(A-1) to (F-1)} except (B-1).
6. The final concentrations of the drugs were 2.56, 1.28, 0.64, 0.32, 0.16, 0.08, µg/ml.
7. Well (A-1) was control (contains only media and cells only).
8. Then plastic strip was used to cover the plate.
9. Plates were incubated at 37ºC for 4 hours.
10. After 4 hours the plate was taken from the incubator.
11. Then the viable parasites were counted by haemocytometer under microscope in each of the wells.

CHAPTER 5
Result

Determination of viable cell count of *Entamoeba histolytica* after treatment with Metronidazole and secnidazole

The study was conducted with raw material of Metronidazole and Secnidazole which was collected from Icepta Pharmaceutical LTD. The *in vitro* sensitivity of Metronidazole and Secnidazole against *Entamoeba histolytica* was observed after treating with different concentration. The different concentrations were (2.56, 1.28, 0.64, 0.32, 0.16, 0.08) µg/ml.

5.1 Mean viable cell count of *Entamoeba histolytica* after treatment with 2.56 µg/ml of metronidazole.

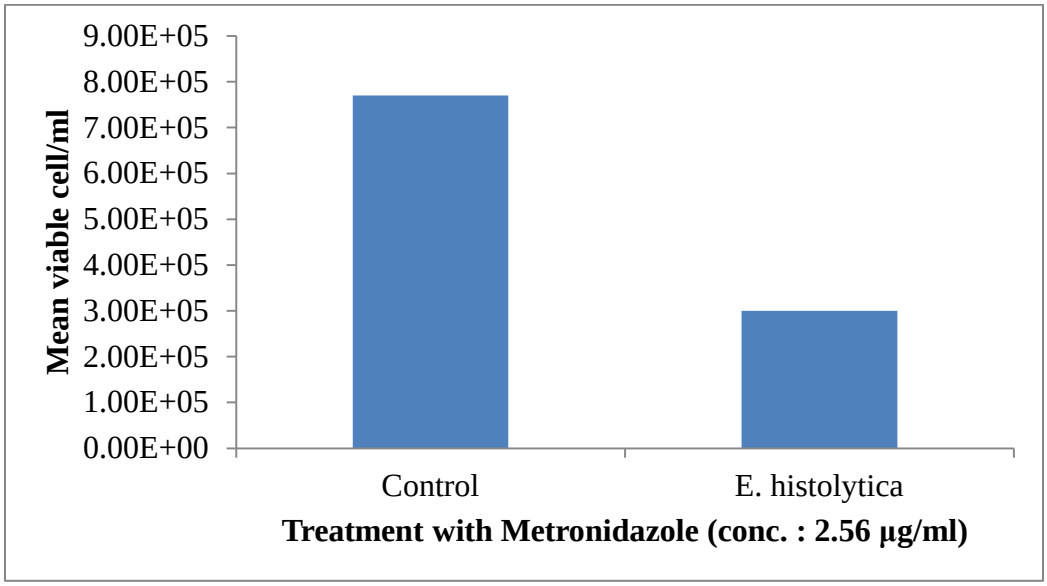

Figure 5. 1 : Mean viable cell count of Entamoeba histolytica after treatment with 2.56 µg/ml of metronidazole.

The bar diagram shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $7.7X10^5$ cell/ml. The viable cell counts of *E. histolytica* after treatment with Metronidazole was $3X10^5$ cell/ml.

5.2 Mean viable cell count of *Entamoeba histolytica* after treatment with 1.28 µg/ml of metronidazole.

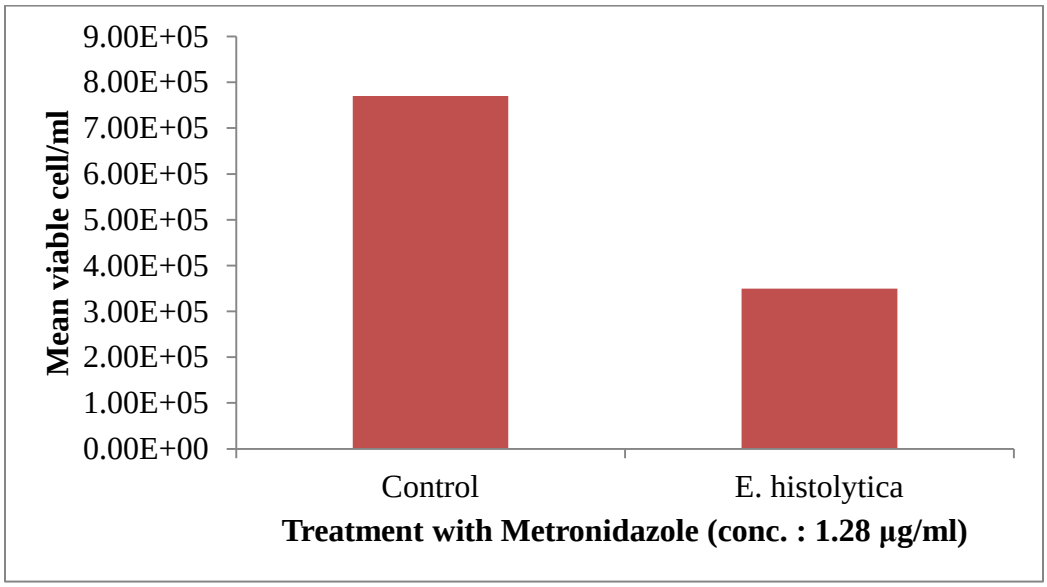

Figure 5. 2 : Mean viable cell count of Entamoeba histolytica after treatment with 1.28 µg/ml of Metronidazole

The diagram shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $7.7X10^5$ cell/ml. The viable cell counts of *E. histolytica* after treatment with Metronidazole was $3.5X10^5$ cell/ml.

5.3 Mean viable cell count of *Entamoeba histolytica* after treatment with 0.64 µg/ml of metronidazole.

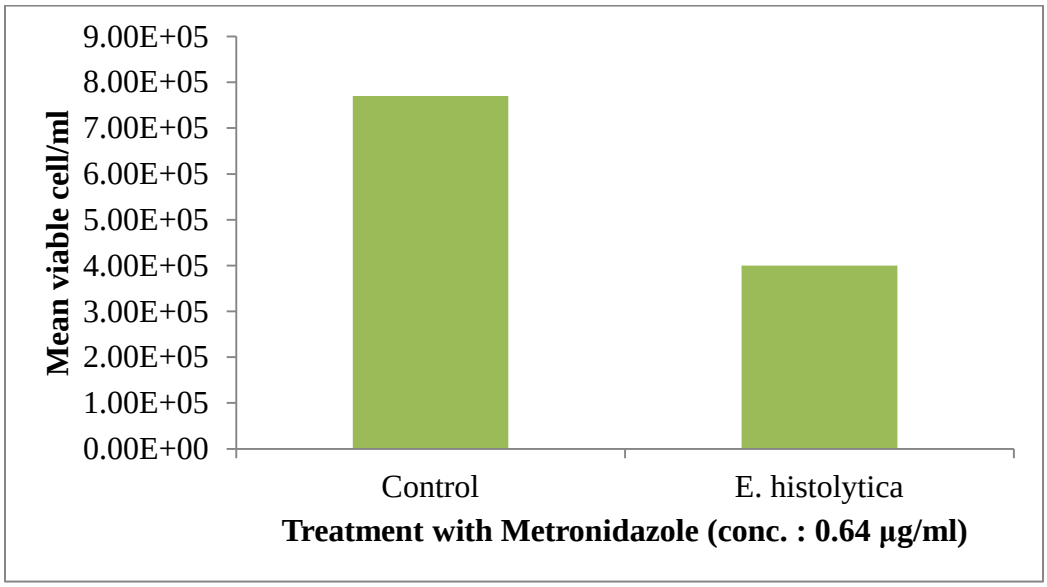

Figure 5. 3 : Mean viable cell count of Entamoeba histolytica after treatment with 0.64 µg/ml of Metronidazole

The figure shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $7.7X10^5$ cell/ml. The viable cell counts of *E. histolytica* after treatment with Metronidazole was $4X10^5$ cell/ml.

5.4 Mean viable cell count of *Entamoeba histolytica* after treatment with (0.32, 0.16, 0.08) µg/ml of Metronidazole

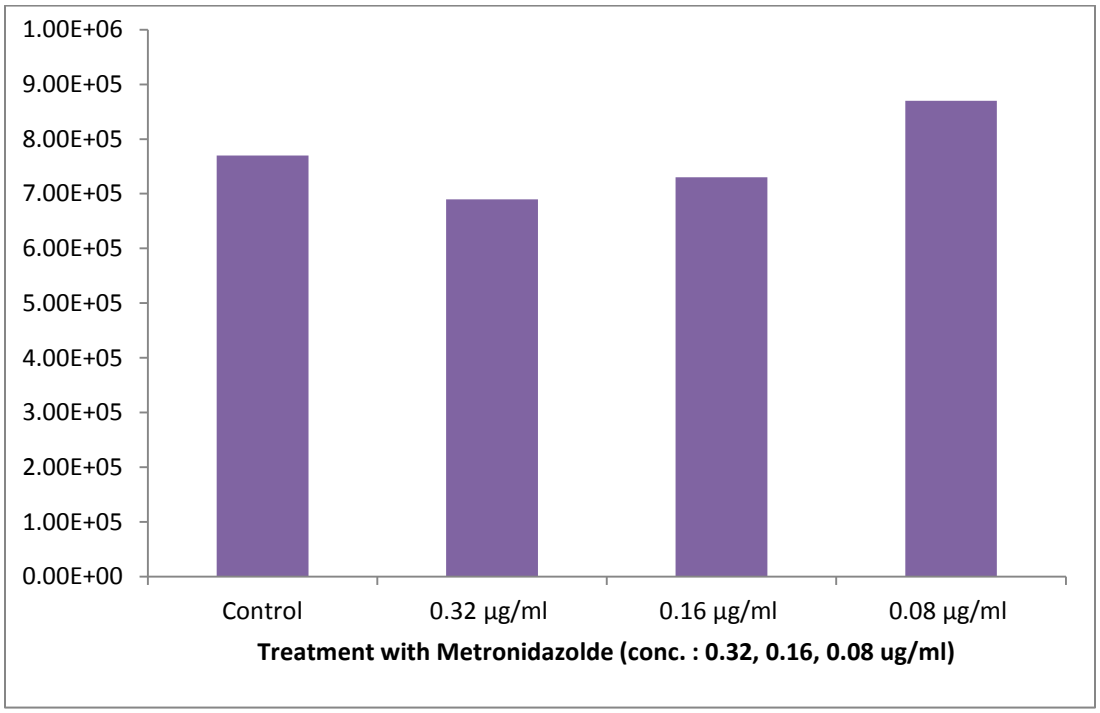

Figure 5. 4 : Mean viable cell count of Entamoeba histolytica after treatment with (0.32, 0.16, 0.08) µg/ml of Metronidazole

The diagram shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $7.7X10^5$cell/ml. The viable cell counts of *E. histolytica* after treatment with Metronidazole with concentration of (0.32, 0.16, 0.08) µg/ml was ($6.9X10^5$, $7.3\ X10^5$, $8.7\ X10^5$) cell/ml respectively.

5.5 Mean viable cell count of *Entamoeba histolytica* after treatment with 2.56 µg/ml of Secnidazole.

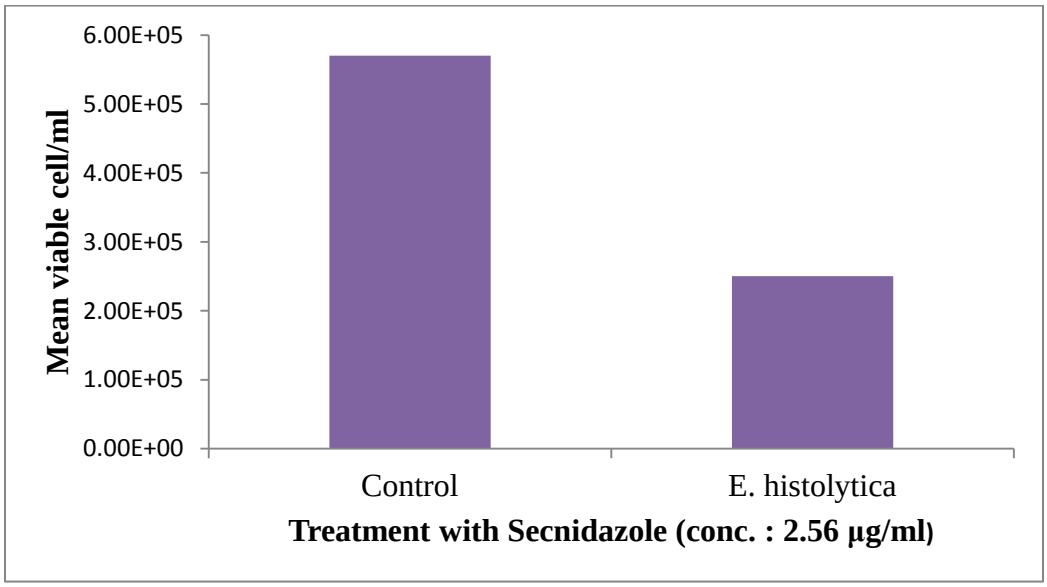

Figure 5. 5 : Mean viable cell count of Entamoeba histolytica after treatment with 2.56 µg/ml of Secnidazole

The figure shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $5.7X10^5$cell/ml. The viable cell counts of *E. histolytica* after treatment with Metronidazole was $2.5X10^5$cell/ml.

5.6 Mean viable cell count of *Entamoeba histolytica* after treatment with 1.28 µg/ml of Secnidazole.

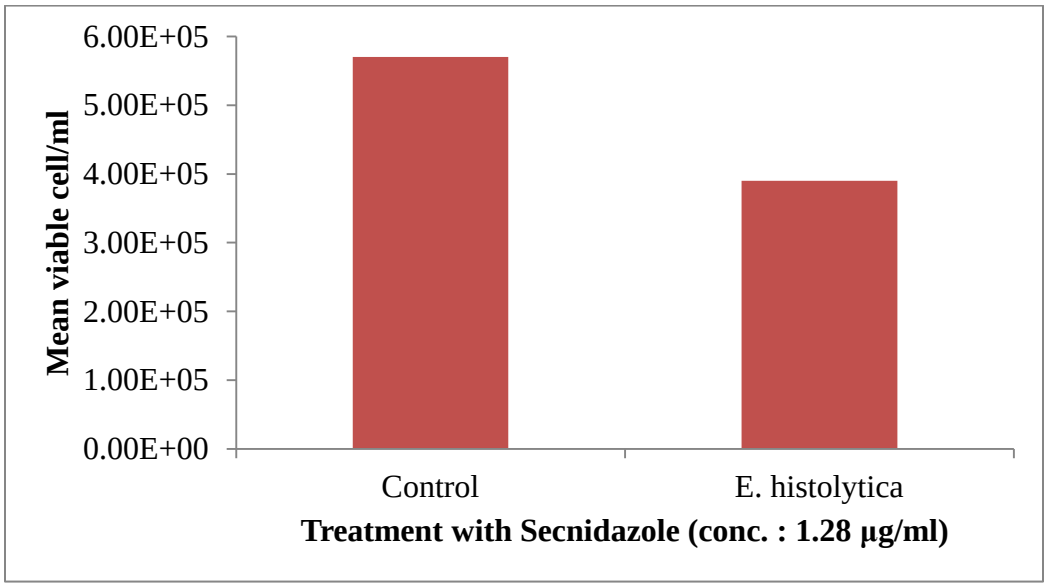

Figure 5. 6 : Mean viable cell count of Entamoeba histolytica after treatment with 1.28 µg/ml of Secnidazole

The bar diagram shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $5.7X10^5$cell/ml. The viable cell counts of *E. histolytica* after treatment with Metronidazole was $3.9X10^5$cell/ml.

5.7 Mean viable cell count of *Entamoeba histolytica* after treatment with 0.64 µg/ml of Secnidazole.

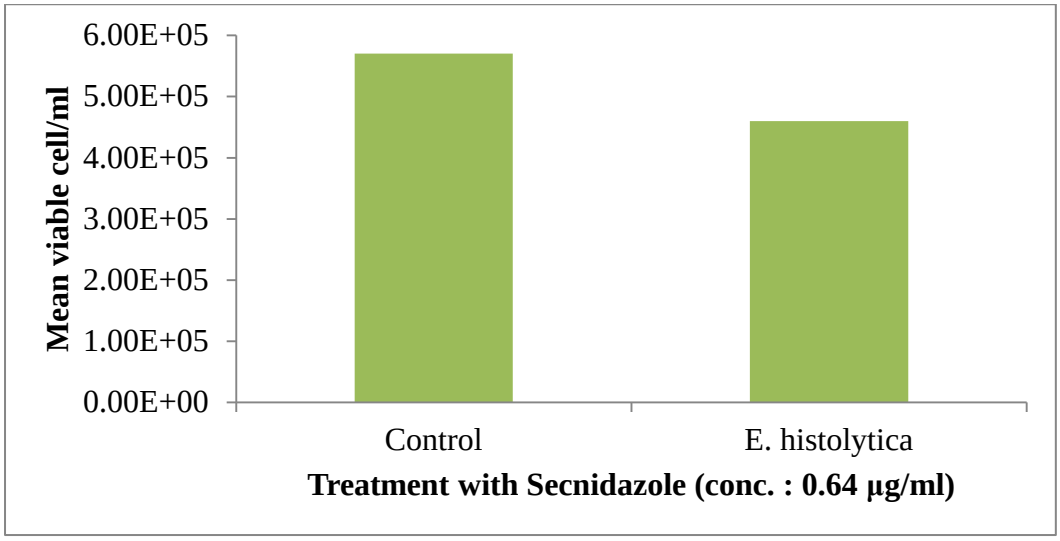

Figure 5. 7 : Mean viable cell count of Entamoeba histolytica after treatment with 0.64 µg/ml of Secnidazole

The figure shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $5.7X10^5$ cell/ml. The viable cell counts of *E. histolytica* after treatment with Metronidazole was $4.6X10^5$ cell/ml.

5.8 Mean viable cell count of *Entamoeba histolytica* after treatment with (0.32, 0.16, 0.08) µg/ml of Secnidazole

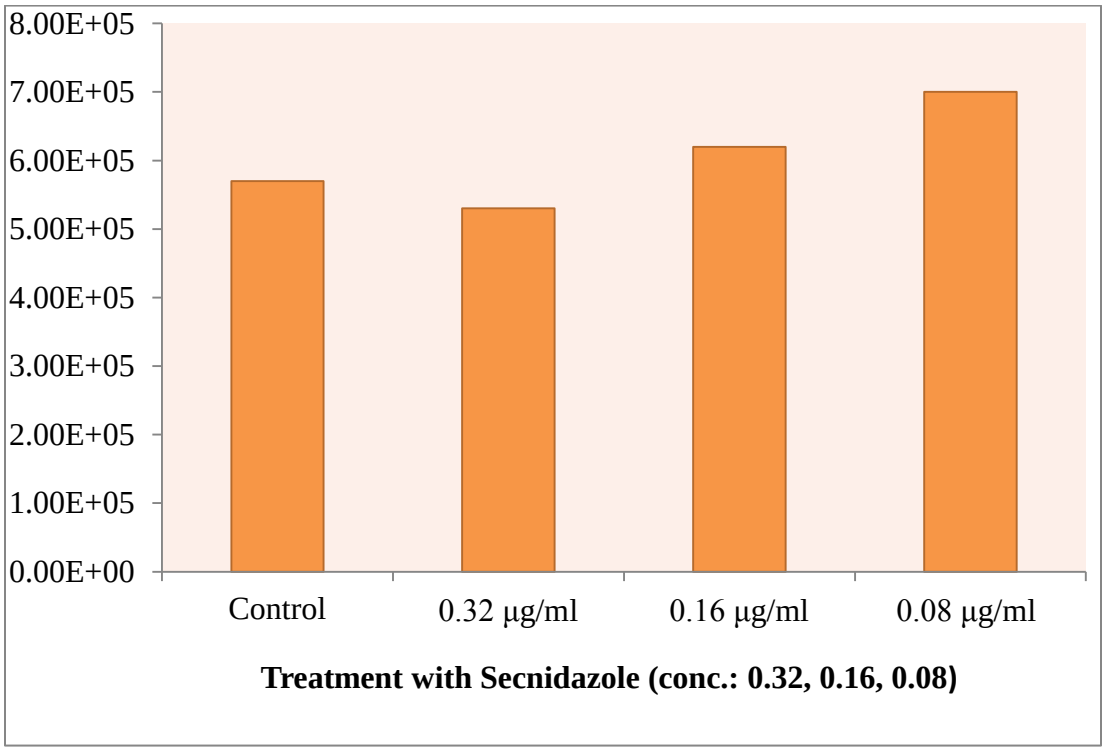

Figure 5. 8 : Mean viable cell count of Entamoeba histolytica after treatment with with (0.32, 0.16, 0.08) µg/ml of Secnidazole

The diagram shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Secnidazole. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $5.7X10^5$cell/ml. The viable cell counts of *E. histolytica* after treatment with Secnidazole with concentration of (0.32, 0.16, 0.08) µg/ml was ($5.3X10^5$, $6.2X10^5$, $7\ X10^5$) cell/ml respectively.

5.9 Mean viable cell count of *Entamoeba histolytica* after treatment with Metrinidazole & Secnidazole Combination (conc.: 2.56, 1.28, 0.64 ug/ml)

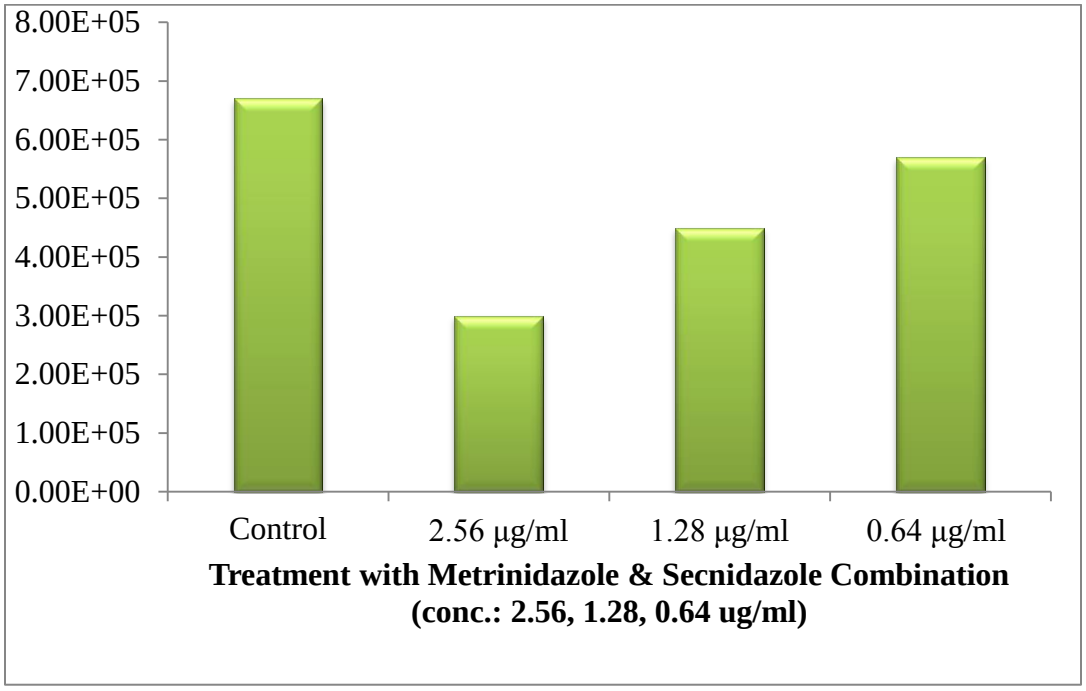

Figure 5. 9 : Mean viable cell count of Entamoeba histolytica after treatment with Metrinidazole & Secnidazole Combination (conc.: 2.56, 1.28, 0.64 ug/ml)

The bar diagram shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metronidazole & Secnidazole Combination. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $7.7X10^5$cell/ml. The viable cell counts of *E. histolytica* after treatment with Metrinidazole & Secnidazole Combination with concentration of (2.56, 1.28, 0.64) µg/ml was ($3X10^5$, $4.5X10^5$, $5.7X10^5$) cell/ml respectively.

5.10 Mean viable cell count of *Entamoeba histolytica* after treatment with Metronidazole & Secnidazole Combination (conc.: 0.32, 0.16, 0.08 ug/ml)

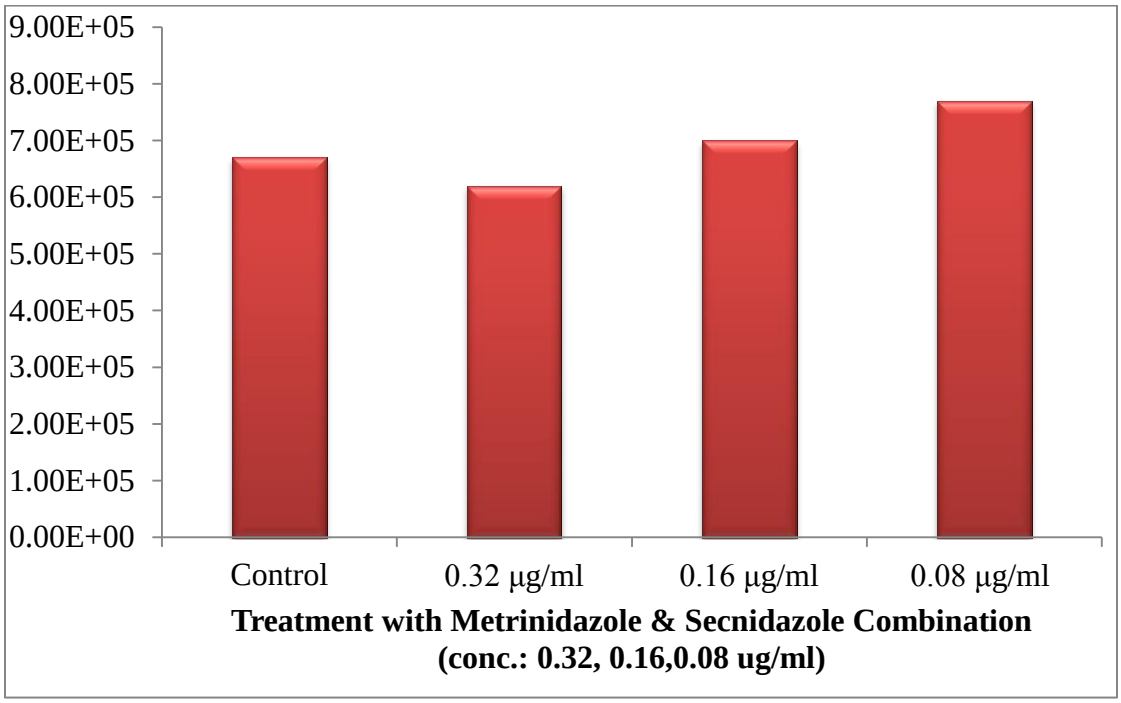

Figure 5. 10 : Mean viable cell count of Entamoeba histolytica after treatment with Metrinidazole & Secnidazole Combination (conc.: 0.32, 0.16, 0.08 ug/ml)

The figure shows the viable cell count from clinical isolates of *Entamoeba histolytica* after treatment with Metrinidazole & Secnidazole Combination. The incubation period was 4 hours. The viable count of *E. histolytica* in control was $7.7X10^5$cell/ml. The viable cell counts of *E. histolytica* after treatment with Metrinidazole & Secnidazole Combination with concentration of (0.32, 0.16, 0.08) µg/ml was ($6.2X10^5$, $7X10^5$, $7.7X10^5$) cell/ml respectively.

CHAPTER 6
Discussion

Entamoeba histolytica is a protozoan pathogen and is best known for its ability to produce amoebic dysentery and liver abscess. Amoebiasis is most prevalent disease in developing countries.

In the present study the *E. histolytica* clinical isolates maintained by *in vitro* cultivation in axenic medium were subjected to drug susceptibility tests against antiamoebic drug Metronidazole & Secnidazole and their combination. The objectives of the study are to investigate the efficacy of Metronidazole and Secnidazole and their combination against *E.histolytica* at different concentrations.

The clinical isolates of parasites were treated with Metronidazole & Secnidazole at different concentrations. In our study we used six different concentrations of both drugs which were 2.56, 1.28, 0.64, 0.32, 0.16 and 0.08 μg/ml. It has been shown from the study that the cell inhibition is maximum at the highest concentration (2.56 ug/ml) of both Metronidazole (61%) & Secnidazole (67.5%).

The viable parasites in Control were $7.7X10^5$ cell/ml and $5.7X10^5$ cell/ml for testing the Metronidazole and Secnidazole respectively.

The maximum cell inhibition of this parasite was found when they were treated with 2.56μg/ml. Cell counts were $3X10^5$ cell/ml & $2.5X10^5$ cell/ml when treated with Metronidazole & Secnidazole respectively.

Viable cell counts of *E. histolytica* were $3.5X10^5$ & $4X10^5$ $6.9X10^5$, $7.3\ X10^5$, $8.7\ X10^5$ cell/ml when different concentrations of Metronidazole (1.28, 0.64, 0.32, 0.16 and 0.08 μg/ml) were used. Viable cells counts of *E. histolytica* were $3.9X10^5$ & $4.6X10^5$, $5.3X10^5$, $6.2X10^5$, $7\ X10^5$ cell/ml when different concentrations of Secnidazole (1.28, 0.64, 0.32, 0.16 and 0.08 μg/ml) were used.

Sarker *et al*. reported *in vitro* sensitivity of different Metronidazole tablets from Bangladeshi pharmaceuticals against clinical isolates of *E. histolytica.* Metronidazole tablets of 12 different brands were randomized from some big and small pharmaceuticals. The parasite count was adjusted to $3x10^6$ parasites mL-1 in a medium. The viable parasites were counted by haemocytometer. No statistical significance was observed in terms of viable parasites with the metronidazole tablets from three big pharmaceuticals at the concentration of 2.3, 3.5 and 4.6 μM when compared with the standard metronidazole (Sarker *et al*, 2008). In our study we adjusted the parasite count to $1x10^6$ cells/ml. Metronidazole and Secnidazole both are from Imidazole

group. We carried out our study by using these drugs which shows same sensitivity against *E. histolytica* after 4 hours of incubation.

In combination treatment it has been shown that viable cell counts of *E. histolytica* were $3.X10^5$ & $4.5X10^5$, $5.7X10^5$, $6.2\ X10^5$, $7\ X10^5$ & $7.7X10^5$ cell/ml when different concentrations of combination (Metronidazole+Secnidazole) (2.56,1.28, 0.64, 0.32, 0.16 and 0.08 μg/ml) were used. Similar mechanism of action of the drug may be the reason that combination of these two drugs didn't show any synergistic effect on *E. histolytica* species.

Further research is required to find out the efficacy of combination drugs with different mechanism of actions on *E. Histolytica* species.

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