Identification of A Novel Anti-Parasitic Compound from Natural Medicinal Sources and their Effect on *Giardia lamblia*

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PREFACE

This Ph.D. thesis was prepared at the Division of Parasitology, ICMR- National Institute of Cholera and Enteric Diseases, India to fulfil the requirements for obtaining a Ph.D. degree. The thesis is titled "**Identification of a Novel Anti-Parasitic Compound from Natural Medicinal Sources and their Effect on *Giardia lamblia***" and the research was conducted at ICMR- National Institute of Cholera and Enteric Diseases, Kolkata, India. The thesis was completed under the direct guidance of Dr. Sandipan Ganguly, Ph.D, Senior Deputy Director (Scientist-F) and Head, Division of Parasitology, ICMR- National Institute of Cholera and Enteric Diseases, Kolkata, India.

This thesis has been solely composed by the candidate and has not been submitted for any other degree, except where specifically acknowledged.

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Date:

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Tapas Haldar

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Dedicated to all those who tirelessly pursue their dreams and strive for a better tomorrow....

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ABBREVIATION

Abbreviation	Meaning
AA	Arachidonic acid
ADG	Andrographolide
ADH	Alcohol dehydrogenase
ALT	Alanine-2-oxoglutarate transaminase
AP	*Andrographis paniculata*
ARF	ADP-ribosylation factor
ATP	Adenosine triphosphate
Ca2+	Calcium ion
Cath-B	Cathepsin B
CO2	Carbon dioxide
COX	Cyclooxygenase
CP	Cysteine protease
DAPI	4', 6-diamidino-2-phenylindole
DCFDA	2'-7'-Dichlorodihydrofluorescein diacetate
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMP	Embden-Meyerhof pathway
ER	Endoplasmic reticulum
ESV	Encystation- specific vesicles
GDH	Glutamate dehydrogenase
GFP	Green fluorescent protein
GPL	Glycerophospholipid
H2O2	Hydrogen peroxide
HK	Hexokinase
K+	Potassium ion
Kb	Kilobase
kDa	Kilodalton
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Milliliter
NADH	Nicotinamide adenine dinucleotide hydrogen
NADP	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
O2	Oxygen
OH	Hydroxyl radical
PBS	Phosphate-buffered saline
PCD	Programmed cell death

Abbreviation	Meaning
PDH	Pyruvate dehydrogenase
PEP	Phosphoenol pyruvate
PFGE	Pulse field gel electrophoresis
PFOR	Ferredoxin oxidoreductase
PI	Propidium iodide
ROS	Reactive oxygen species
SEM	Scanning electron microscope
µl	Microliter
µM	Micromole
МЬ	Megabase

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ABSTRACT

Identification of a novel Anti-Parasitic Compound from Natural Medicinal sources and their effect on *Giardia lamblia*

Giardia lamblia is one of the most frequent protozoan parasite causes giardiasis worldwide. According to WHO reports annually 280 million people have been infected globally. Giardiasis is becoming an increasingly prevalent issue worldwide, particularly in developing country, as it continues to emerge and spread within human populations. In developed countries, the prevalence rate of giardiasis is between 2-5%, while in developing countries ranges from 20-30%. The disease is typically transmitted indirectly through the consumption of food or water that has been contaminated with cysts, or through person-to-person contact, especially among individuals living in unhygienic conditions. However, the present medications for Giardiasis treatment often come with numerous side effects, while the growing problem of drug resistance adds a significant complication. It is required to develop both new drugs and treatment strategies that can enhance therapeutic outcomes and effectively address drug-resistant cases in clinical settings.

Today, medicinal plants are at the forefront of primary healthcare, valued for their accessibility, acceptability, and affordability. With a rich history of use spanning millennia, evidenced by their presence in ancient medical systems like Ayurveda, Unani, and Siddha, medicinal herbs continue to play a crucial role. India, known for its biodiversity, houses over 45,000 plant species, potentially making it the largest producer of medicinal herbs. Among these, the *Andrographis paniculata* (*A. paniculata*) plant shines with its exceptional therapeutic properties, experiencing a surge in demand in recent years. This indigenous herb holds not only economic importance but also serves as a potent remedy, highlighting its significance both economically and medicinally. The pharmacological effects of *A. paniculata* have been studied using both crude extracts and isolated bioactive compounds. While the crude extract showed notable effects, focusing on the major bioactive compound, andrographolide, offers a clearer understanding, especially regarding mechanisms of action. Andrographolide exhibits diverse activities, including anticancer, antidiabetic, anti-inflammatory, and hepatoprotective effects, which could benefit a large population. This study primarily explores the medicinal and pharmacological effects of andrographolide, targeting giardiasis.

In this study, we explored the in-vitro potential of *A. paniculata* (*Ap*) leaf extract and its active compound andrographolide (ADG) as a possible alternative treatment for giardiasis. Natural compounds show promise in the search for more potent antigiardial agents. After 24 hours of incubation, the test results revealed *Ap* extract and ADG demonstrated an IC_{50} value of 51.26µg/ml and 4.99µM. We observed a substantial degradation of DNA, alteration in morphology, inhibition in adherence, ROS generation and inhibition of the cell cycle in *Giardia* trophozoites. It also downregulates many genes that are related to pathogenesis. The in vivo results showed a favorable response to ADG treatment at 20 mg/kg body weight in infected mice, with no significant toxicity observed on the intestinal cell line. This encouraging outcome supports the formulation of a new drug development strategy against giardiasis. This approach aims to offer a natural therapeutic solution for giardiasis, minimizing side effects and reducing the risk of drug resistance.

5.7.24

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CHAPTER-I

INTRODUCTION

1.1. Introduction

Giardia lamblia (*Giardia duodenalis* or *Giardia intestinalis*) is a flagellated protozoan parasite and the causative agent of giardiasis, a significant public health concern. *G. lamblia* is the most common protozoal infection in humans and is a major cause of waterborne and foodborne diarrheal diseases. It is estimated that approximately 280 million cases of giardiasis are diagnosed annually worldwide. The prevalence of giardiasis in humans varies from 2–3% in developed countries to as high as 30% in developing countries (Ankarklev et al. 2010). The life cycle of *G. lamblia* is direct and consists of two stages: the trophozoite, responsible for replication, and the cyst, which serves as the infective stage (Ryan et al. 2013). The disease is typically transmitted indirectly through the consumption of food or water that has been contaminated with cysts or through person-to-person contact, especially among individuals living in unhygienic conditions. Giardiasis can either be asymptomatic or result in symptoms such as diarrhoea, vomiting, flatulence, anorexia, and crampy abdominal pain during its acute phase (Escobedo et al. 2018). In addition to gastrointestinal symptoms, it may also lead to extra-intestinal manifestations such as fever, maculopapular rash, pulmonary infiltrate, lymphadenopathy, polyarthritis, urticaria and growth retardation. Giardiasis primarily affects children, and one of its severe consequences is malnutrition and growth inhibition resulting from early or recurrent infections (Mørch et al. 2020).

Transmission of *Giardia* is common among individuals with poor hygiene practices and can lead to asymptomatic, acute, or chronic illness, influenced by various factors such as patient age, previous exposure, parasite load, virulence, and host immune response (Leung et al. 2019). Despite adequate sanitation, protection against giardiasis remains insufficient, and there are currently no biological prophylactic methods available to prevent *Giardia* infections, necessitating reliance on treatment (Argüello-García et al. 2020). An effective and approved human vaccine against giardiasis remains unavailable, making pharmacotherapy the sole option for treating the condition. In low-prevalence settings, treatment of confirmed giardiasis cases is always advised to alleviate symptoms and expedite recovery. Additionally, prompt treatment may lower the likelihood of post-infectious complications and help contain the spread of the infection. A Cochrane review conducted in 2012 examined a mere 19 randomized controlled trials (RCTs) comparing the efficacy of metronidazole (MTZ) administered over 5–10 days with various alternatives, including single-dose MTZ, tinidazole, albendazole (ABZ), mebendazole, and nitazoxanide (Moerch et al. 2008). The primary conclusion drawn was that MTZ and ABZ exhibited similar effectiveness, with ABZ demonstrating fewer side effects and

a simpler drug regimen. However, due to the limited number of trials involving other medications, definitive conclusions could not be reached.

Irrespective of the drug and treatment regimen employed, achieving a 100% parasitological cure rate in giardiasis cases is rare, indicating the possibility of treatment failure in some instances. Similar to bacteria, prolonged use of antimicrobials can lead to the development of resistance in parasites. The term "treatment refractory" is commonly employed when discussing clinical infections, as there can be various reasons for treatment failure, including the possibility that the infecting *Giardia* isolate is genuinely drug resistant. Clinically, cases of giardiasis refractory to treatment have been noted by clinicians for decades. However, with enhanced diagnostic techniques and increased patient cases of *Giardia* resistant to MTZ have recently been extensively documented in low-prevalence settings (Muñoz Gutiérrez et al. 2013). The susceptibility of *Giardia* to MTZ treatment, which persisted for five decades, is now being replaced by a rising trend in resistance. Additionally, cases of refractory giardiasis have been reported even after treatment with all other available drugs. The prevalence of refractory infections from Asia was found to be higher (70%) in this study compared to infections from other areas. Both assemblages A and B were responsible for the resistant illnesses seen in the Spanish investigation, indicating that a number of circulating strains may be resistant to nitroimidazole (Lecová et al. 2018). In line with this, nine (19%) of the 47 isolates from a recent Czech investigation that genotyped them were from patients who were clinically resistant to MTZ.

Andrographis paniculata (Burm. F.) Nees, a member of the Acanthaceae family, is a plant that is commonly found in several Asian countries including China, India, Thailand, and Sri Lanka (Hossain et al. 2014, Akbar et al. 2011). It has a long history of medicinal use in both Indian and Oriental medicine. Ancient Ayurvedic texts have highlighted its potential as an herb for addressing neoplasms and have documented at least 26 Ayurvedic formulations that incorporate it in the treatment of liver disorders (Okhuarobo et al. 2014; Mishra et al. 2007). The plant, commonly referred to as the "king of bitters," is widely recognized for its highly bitter characteristics and has been traditionally employed as a treatment for various ailments, including the common fever, cold, dysentery, tonsillitis, liver ailments, diarrhoea, inflammation, herpes, and many others(Hossain et al. 2014). Mainly the leaves and roots are used for medicinal purposes. The aerial part of the plant contains a significant number of chemical constituents, primarily lactones, diterpenoids, diterpene glycosides, flavonoids, and flavonoid glycosides (Jarukamjorn & Nemoto, 2008).

The species has been extensively used for therapeutic purposes and is effective in treating a wide range of diseases such as antidiarrheal (Gupta et al. 1993), hepatoprotective (Trivedi & Rawal 2001), anti-inflammatory (Chiou et al. 2000; Sheeja et al. 2006), anticancer (Kumar et al. 2004), antihepatitis (Sharma et al. 1991), anti-HIV (Calabrese et al. 2000), antimicrobial antimalarial (Misra et al. 1992), antioxidant (Kamdem et al. 2002), antihyperglycemic (Subramanian et al. 2008), cardiovascular (Tan & Zhang 2004), cytotoxic (Geethangili et al. 2005), immunostimulatory (Iruretagoyena et al. 2005).

The plant contains various active compounds, such as diterpene lactones, flavonoids, and polyphenols, as reported in studies (Okhuarobo et al. 2014). Andrographolide (ADG), a diterpenoid lactone with the chemical formula $C_{20}H_{30}O_5$, serves as the primary compound responsible for the plant's therapeutic properties (Chao and Lin. 2010). It is primarily concentrated in the leaves of the plant and can be readily isolated from plant crude extracts as a crystalline solid (Varma et al. 2011). ADG demonstrates an extremely broad spectrum of biological activities. Recent reports suggest that it possesses anti-tumour, cardio-protective, anti-HIV, antioxidant, anti-inflammatory, immunomodulatory, antibacterial, cytotoxic, neuroprotective, and hepatoprotective properties (Li et al. 2022). Furthermore, it has been shown to exhibit antimicrobial activity against bacteria and viruses. Since ancient times, medicinal herbs and their derivatives have been widely utilized for promoting health and treating chronic, non-life-threatening ailments (Lalle & Hanevik, 2018). Consequently, further research into medicinal plants or herbs as alternative treatments for giardiasis is warranted. It is believed that natural compounds have therapeutic potential and are less harmful than manufactured drugs.

1.2. Objectives

The aim of this study is to investigate the antigiardial activity of *A. paniculata* against *Giardia* trophozoites. While the plant has demonstrated antibacterial, antifungal, and antiviral activities, but antigiardial effects have not yet been reported. The primary objective of this proposed study is to evaluate the impact of *A. paniculata* and its active compound against *Giardia* trophozoites through both in vitro and in vivo studies. Additionally, the study aims to examine the gene expression changes following treatment with andrographolide during pathogenesis. For this purpose, following objectives will be considered:

I. Studies the cytotoxic activity of plant extract against *Giardia* in vitro.

II. Studies the antigiardial activity of Andrographolide (An active compound of *A. paniculata*) in-vitro.

III. Studies the different gene expression level after the treatment of Active compound (Andrographolide).

IV. Studies the antigiardial activity of the active compound of *Andrographis paniculata* (Andrographolide) *in vivo*.

CHAPTER-II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Introduction

Giardia lamblia (*G. lamblia*) frequently causes diarrhoea in humans and other mammals worldwide. It is responsible for one of the most common parasitic infections globally, affecting an estimated 280 million individuals with symptomatic infections every year (Adam 2001). Unlike other eukaryotes, *Giardia* lacks conventional mitochondria, Golgi bodies, and peroxisomes. The onset of giardiasis symptoms, which include watery diarrhea, abdominal pain, irritable bowel syndrome, nausea, vomiting, weight loss, typically occurs 6-15 days after infection (Farthing 1997). While malnourished children and immunocompromised individuals are more prone to experiencing severe symptoms of the disease, the standard treatment for giardiasis involves the use of metronidazole or other nitroimidazoles. *Giardia* do not invade the gut nor secrete any known toxins, but recent research suggests that it can increase intestinal permeability by promoting apoptosis of the intestinal epithelial cells (Scott et al. 2002; Singer & Nash 2000). *Giardia* infections in farm animals can also lead to reduced economic yield (O'Handley et al. 2001). Although the infection is typically chronic, almost half of the cases are asymptomatic and may resolve on their own (Farthing 1997). Some studies suggest that a previous *Giardia* infection may be associated with gastrointestinal disorders such as irritable bowel syndrome, but the symptomatology can vary from person to person (Hanevik et al. 2009). *Giardia* is a protist genus that belongs to the diplomonads, a group of binucleated flagellates now classified under the supergroup Excavata (Simpson 2003). As one of the most divergent eukaryotes studied to date, it offers unique opportunities to gain a better understanding of the fundamental pathways that characterize eukaryotic cells and new molecular mechanisms within them. *G. lamblia* serves as an excellent model system due to its unusual ultrastructure, simple in vitro differentiation, sequenced genome, and bacterial-like metabolism (Lauwaet et al. 2007). The reductive processes and parasitic lifestyle of *Giardia* are critical for its survival, shedding light on its evolution. *Giardia* is a microorganism that has attracted considerable scientific interest due to its unique evolutionary position and ability to survive in two very different environments. In humans, *Giardia* exists as a flagellated trophozoite form that inhabits the small intestine. Meanwhile, the cyst form of the organism can withstand extreme conditions and persist in cold freshwater. One of the most intriguing aspects of *Giardia* is its sensitivity to oxygen levels. This microaerophilic organism is unable to tolerate high levels of oxygen pressure and has been found to thrive in areas where oxygen concentrations are as low as 60µM

in the upper lining of intestinal cells. Additionally, *Giardia* lacks several enzymes that are typically involved in detoxifying reactive oxygen species (ROS), including superoxide dismutase, catalase, peroxidase, and glutathione reductase. These unique features make *Giardia* an important subject of study for researchers seeking to better understand the biology of early-evolving organisms and the mechanisms by which microorganisms adapt to their environments. Recent research suggests that *Giardia*, which is highly sensitive to oxygen tension, prefers to reside in the proximal small intestine rather than the colon, which has a higher redox buffering capacity (Mastronicola et al. 2011). The exact mechanism by which the parasite helps in the detoxification of ROS produced during oxidative stress remains unknown. Cysteine, a vital amino acid for *Giardia*, is not produced de novo nor from cystine, and is believed to be transported inside the cell via passive diffusion, although some active transport has also been observed (Lujan et al. 1994). The importance of free thiol groups on the surfaces of trophozoites was demonstrated by the toxicity of thiol-blocking agents that were unable to penetrate intact cells (Gillin et al. 1984). *Giardia* is a micro-aerophilic parasite that infects all vertebrates, lacks conventional mitochondria, but contains mitosomes (apparent relics of mitochondria). *Giardia* is a eukaryotic organism that displays both typical and prokaryotic-like characteristics. It possesses a distinct nucleus and nuclear membrane, endomembrane system and cytoskeleton, but some key metabolic enzymes and SSU rRNA resemble those of prokaryotes (Svard et al. 2003). Although there is controversy surrounding this, many researchers believe that *Giardia*, having diverged during the transition of mitochondrial acquisition, provides a valuable model for understanding the evolution of eukaryotic cells (Thompson 2004). The sequencing of the *Giardia* genome has greatly enhanced our understanding of this fascinating organism.

2.2 History of the discovery of *Giardia*

Discovered by the Dutch microscopist Anton Van Leeuwenhoek in 1681 from his own stool, *G. lamblia* is a unicellular flagellated intestinal protozoan that has a rich history of 300 years, but remains a mystery in the field of biology. The morphology of *Giardia* was first described by Lambl in 1859 and 1860, as well as by Cunningham in 1881. Since the initial characterization of the organism, the nomenclature within this group has remained a consistent source of confusion, with a lack of consensus that endures to the present day. The naming issues pertain to both the genus and species, with two different names, *Giardia* and *lamblia*, currently in use to refer to these organisms. Kunstler 1882 originally proposed the name *Giardia* as the genus name. However, Blanchard 1888 recommended the name *lamblia* in honor of Lambl

1859. Eventually, in 1915, Stiles officially named this parasite *G. lamblia*.

2.3 Systemic position of *Giardia*

The conventional approach to classifying living organisms has been to divide them into prokaryotes or eukaryotes, and some proponents still advocate for this approach (Mayr, 1998). The most accepted classification system of living organisms is divided into three groups Archaea (archaebacteria), Bacteria (eubacteria), and Eukarya (eukaryotes). These domains can then be further subdivided into various kingdoms based on shared characteristics. In accordance with the classification system, it is evident that *G. lamblia* is typically categorized as a protozoan, belonging to the subset of unicellular eukaryotes. The organisms belong protozoans have been classified based on their morphology into various groups, including ciliates, flagellates, amoebae (rhizopods), and sporozoans. Initially, *G. lamblia* was grouped together with other flagellated protozoans such as parabasalids (*Trichomonas vaginalis*), kinetoplastids (*Leishmania spp*.), and Dientamoeba (*Dientamoeba fragilis*) due to its physical appearance. *Giardia* is placed in the order Diplomonadida, which have four flagella, two nuclei, two karyomastigonts no Golgi complex and no mitochondria. *Giardia*, a member of the family Hexamitidae, displays bilaterally symmetrical morphology with two nuclei, eight flagella and also it may exhibit parabasal bodies and axostyles and it has the ability to form cysts.

According to a phylogenetic tree constructed based on the cpn60 gene, which is believed to have mitochondrial origins, *Giardia* appears to be an eukaryotic organism that diverged early in evolution (Figure 2.2) (Germot et al. 1996; Roger et al. 1998). Additionally, evidence of lateral gene transfer is provided by the iron-dependent hydrogenases found in *G. lamblia* (Nixon et al. 2002 & 2003).

G. lamblia the gene triose phosphate isomerase recently been discovered as a valuable genetic marker for studying the population genetics, as indicated by studies conducted by Lin et al. (2013). The available data mostly support the idea that *G. lamblia* is earliest diverging eukaryotes.

Systematic Position (Source: -López et al., 2022)

PHYLUM: Metamonada

CLASS: Fornicata

ORDER: Diplomonadida

FAMILY: Hexamitidae

GENUS: *Giardia*

SPECIES: *lamblia*

Figure **2.1**: Classification of *Giardia*

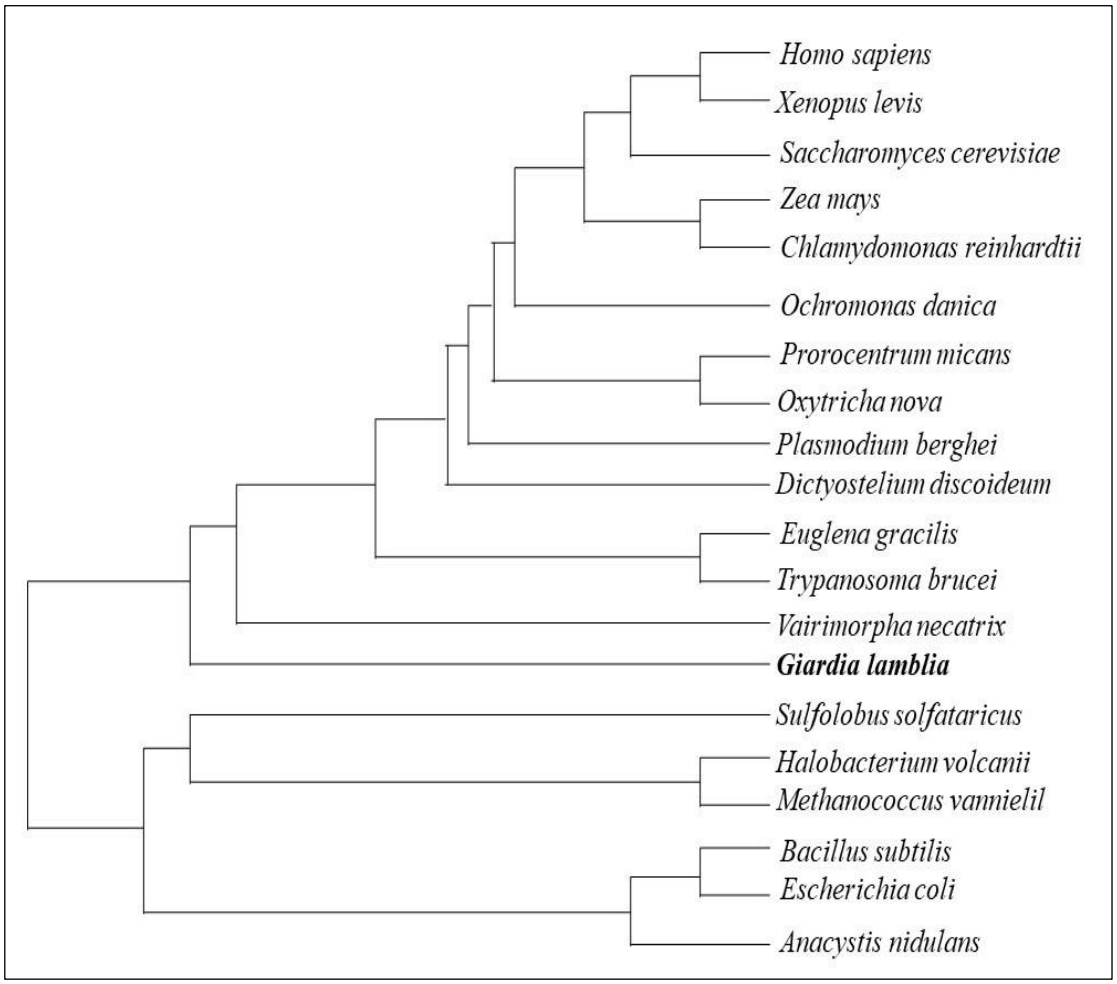


Figure 2.2: Multikingdom tree inferred from 16S-like rRNA

Interestingly, over ten years ago, investigations into *Giardia*'s population genetics within endemic communities, where the parasite transmission occurs at a high rate, revealed signs of sporadic genetic exchange (Meloni et al. 1995). Through allozyme electrophoresis, Meloni & colleagues 1995) observed several different banding patterns in several isolates of *Giardia*. If these patterns are indeed indicative of the underlying genotypes of the isolates, then it suggests that *G. lamblia* has a functional diploid nature and that sexual reproduction or recombination must have taken place at some point to create the apparent heterozygotes. Recent population genetic research (Cooper et al. 2007) and molecular analyses have proved that the idea that *G. lamblia* is not limited in clonal asexual organism. Research has shown that *Giardia* has retained certain aspects of meiotic machinery, including the capacity for chromosomes to undergo some recombination site and crossover sites. (Ramesh et al. 2005; Poxleitner et al. 2008).

The notion that recombination occurs between assemblages of *G. lamblia* implies that these assemblages have a common gene pool, raising concerns about whether it is valid to classify genetically distinct *G. lamblia* assemblages as a species complex. Despite the absence of observable sexual reproduction in *Giardia*, the population genetics studies suggest that sexual reproduction may occur infrequently, which could explain the lack of observations (Meloni et al. 1995; Cooper et al. 2007; Ankarlev et al. 2010). *Giardia* benefits from genetic exchange as it enables the organism to adapt to challenging conditions, such as exposure to anti-*Giardial* drugs or competition with other strains in environments where mixed infections are prevalent. This advantage allows *Giardia* to respond to selective pressures and maintain its survival. However, the occurrence of genetic exchange may not be common, and additional research is necessary, especially in areas where infection rates are high, to better understand its frequency. This information comes from Hopkins et al. 1999 study, and the evolutionary benefits of genetic exchange in *Giardia* were highlighted. The available data suggests that the genetic assemblages of *Giardia* remain consistent with their geographic and host distribution, indicating that any genetic recombination does not occur at the assemblage or species level. However, recent research has revealed genetic recombination between different *G. lamblia* isolates (Caccio et al. 2008). This discovery emphasizes the need for caution when interpreting results based on a single locus. To accurately study the phylogeny of *G. lamblia* and conduct genotyping studies for epidemiology, researchers must use multiple loci to account for potential genetic recombination.

2.4 Genotypes of *G. lamblia*

The use of molecular classification tools has played a crucial role in comprehending the pathogenesis and host specificity of *Giardia* isolates acquired from diverse mammalian sources (Table 2.1). In 1983, Bertram conducted the first investigation of molecular distinctions among *G. lamblia* isolates. In this study, five axenized isolates were analyzed using a zymodeme assessment that involved six metabolic enzymes. The isolates were obtained from three human sources, one guinea pig source, and one cat source. In 1985, Nash conducted a study that involved the analysis of 15 isolates through restriction fragment length polymorphism using random probes. As a result, three groups were identified, with group III being distinct enough from groups I and II to suggest the possibility of it being a separate species. Since then, various other molecular classification investigations have been conducted. Although researchers have also explored the patterns of chromosomes using pulsed-field gel electrophoresis (PFGE) (Campbell et al. 1994; Korman et al. 1992; Isaac-Renton 1993), the usefulness of such patterns in classification is constrained by the frequent incidence of chromosome rearrangements (Adam 1991; Le Blancq et al. 1991). Similarly, the surface antigen classification described in the study Nash et al. 1985 showed that there is variation in antigens within variant-specific proteins (VSPs) (Nash et al. 1990; Adam 2001). While these studies have been valuable, the semi-quantitative nature of the data restricts the conclusions that can be drawn from them. The comparison of gene sequences such as glutamate dehydrogenase (GDH), small-subunit triosephosphate isomerase, and rRNA is a crucial tool for understanding the genetic diversity and evolution (Baruch et al. 1996; Franzen et al. 2013) and conducted a comparison of the polyadenylated transcriptomes from assemblages A, B, and E, and have described their characteristics. The study found that the analysis of genome content is not dependent on having closed genomes of megabase size; rather, if sequencing libraries are random, then 3-4x coverage is deemed sufficient to sample over 95% of the genome. Franzen et al. 2009 reported on the genome properties of two strains of Assemblage B, GS and 12BC14, as well as one isolate of Assemblage E, P15. These three genomes were generated using 454 pyrosequencing at approximately 16x coverage. According to the study's major findings, WB and GS have a protein coding region with around 78% amino acid identity and a low number of genes that are unique to one genome or the other. Despite being a non-human isolate, P15 was found to have a highly similar gene complement and large gene families compared to the Assemblage B strains. Commonly found among the multigene families of *Giardia* are high-cysteine proteins, VSPs, coiled-coil proteins, NEK kinases, ankyrin-repeat protein, spindle pole proteins, and

endonucleases. The sampling process for each of the three isolates is considered reliable as the metabolic pathway components are similar and conserved groups such as structural proteins, ribosomal, and tRNA synthetasesare present in the expected quantities. However, the variation of antigen differs significantly, which is likely the cause of their contrasting epidemiological profiles.

Species	Hosts	Morphological characteristics	Trophozoite dimensions length/width (μm)
G. duodenalis (=Assemblage A)	Wide range of domestic and wild mammals including humans	Pear-shaped trophozoites with claw-shaped median bodies.	12–15/6–8
G. agilis	Amphibians	Long, narrow trophozoites with club-shaped median bodies.	20–30/4–5
G. muris	Rodents	Rounded trophozoites with small round median bodies.	9–12/5–7
G. ardeae	Birds	Rounded trophozoites, with prominent notch in ventral disc and rudimentary caudal flagellum. Median bodies round-oval to claw shaped.	~10/~6.5
G. psittaci	Birds	Pear-shaped trophozoites, with no ventro-lateral flange. Claw-shaped median bodies.	~14/~6
G. microti	Rodents	Trophozoites similar to *G. duodenalis*. Mature cysts contain fully differentiated trophozoites.	12–15/6–8
G. enterica (=Assemblage B)	Humans and other primates, dogs, some species of wild mammals	Pear-shaped trophozoites with claw-shaped median bodies.	12–15/6–8
G. canis (=Assemblage C/D)	Dogs, other canids	Pear-shaped trophozoites with claw-shaped median bodies.	12–15/6–8
G. cati (=Assemblage F)	Cats	Pear-shaped trophozoites with claw-shaped median bodies.	12–15/6–8
G. bovis (=Assemblage E)	Cattle and other hoofed livestock	Pear-shaped trophozoites with claw-shaped median bodies.	12–15/6–8
G. simondi (=Assemblage G)	Rats	Pear-shaped trophozoites with claw-shaped median bodies.	12–15/6–8

Table 2.1: *Giardia* Genotypes; Adapted from –*Giardia*: A Model Organism by Lujan & Svard ‘2011.

2.5 Life cycle

The life cycle of *G. lamblia* involves two distinct stages, the infective cyst stage and the vegetative trophozoite stage. Typically, infection occurs when individuals ingest cysts present in water contaminated with fecal matter or occasionally through food consumption (Fig. 2.3) (Esch et al. 2013). The process of excystation, which involves the activation of cysts, is initiated by the low pH levels in the stomach's gastric acid (Bingham et al. 1979). Later, the excystation process is further triggered by the proteolytic activity and slightly alkaline pH present in the duodenum (Rice et al. 1981). The presence of cysteine protease is essential for the successful infection of *G. lamblia*, as it acts on the gap between the trophozoites and the intestinal wall during excystation. This protease is stored in the peripheral vesicles of the trophozoites (Ward et al. 1987). The newly formed parasites after excystation divide quickly two similar binucleated trophozoites and then attached and colonize into the human intestine in a target manner. The trophozoites of *G. lamblia* are capable of swimming in the lumenal fluid using their flagellates (Ghosh et al. 2001) and also exhibit the ability to adhere to mucus strands during in vitro, in vivo (Adam 1991). In order to attach to intestine wall, the trophozoites with the help of ventral adhesive disc they can penetrate the mucus layer (Nemanic 1979). Once attached, they obtain nutrients from the host gut by utilizing micropinocytotic vesicles and the trophozoites multiply through binary fission while in this position. The trophozoites undergo encystation as they migrate downward because they cannot survive outside the host. Encystation is initiated by the high bile concentration and elevated pH present in the intestinal lumen, which also induces cyst antigenic factor (Gillin et al. 1987; Reiner et al. 2008). The most noticeable transformation that occurs during encystation is the gradual rounding up of the trophozoites detachment, loss of mobility, and the development of retractile properties (Fig. 2.4).

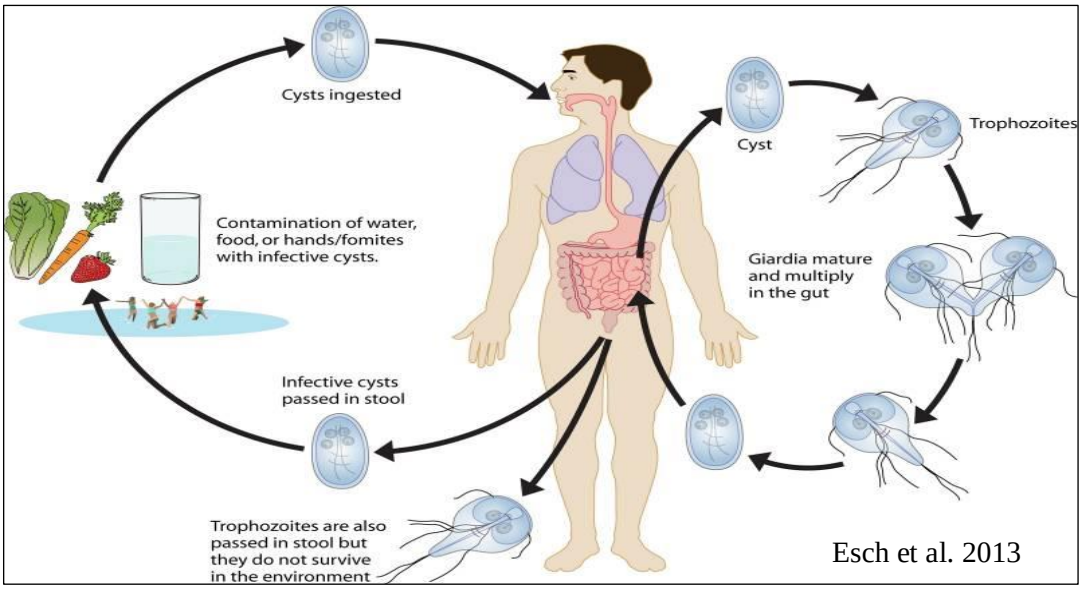


Figure 2.3: Life cycle of *Giardia sp.*

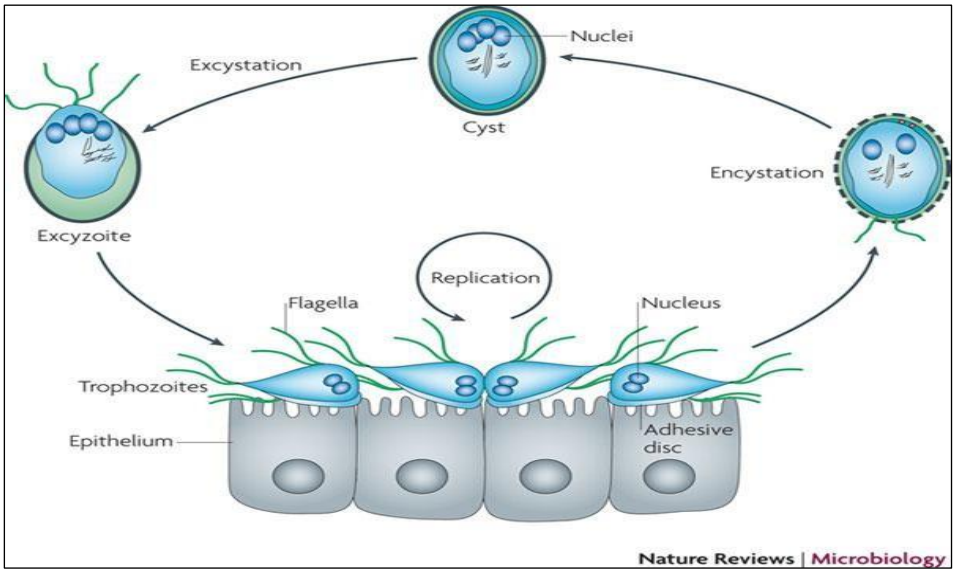


Figure 2.4: *Giardia* cysts are exposed to gastric acid during their passage through the host's stomach, triggering excystation.

2.5.1 Trophozoite Structure

The *G. lamblia* trophozoites structurally pear shaped, measuring the length around 12 to 15 µm and width is 5 to 9 µm. They possess a cytoskeleton that comprises a median body, a ventral disk, and number of flagella four pair (anterior, posterior, caudal, and ventral) (Figure 2.5). The two nuclei of the trophozoites lack nucleoli and are situated at the anterior end, showing symmetry with respect to the long axis. The cytoplasm of the trophozoites contains lysosomal vacuoles, ribosomal granules, and glycogen granules. Although the presence of Golgi complexes in vegetative trophozoites is not confirmed, they become visible during the encystation process (Gillin et al. 1996). Nevertheless, studies have shown stacked membranes that suggest the existence of Golgi complexes (Soltys et al. 1996; Lanfredi-Rangel et al.1999).

2.5.2 Cytoskeleton and Ventral Disc

G. lamblia trophozoites attach to the mid-jejunum region of the small intestine using their ventral adhesive disk to obtain nutrients and prevent transport beyond that region. The ventral disk is a crucial part of their cytoskeleton that contains various contractile proteins, including actinin, alpha-actinin, myosin, and tropomyosin, which contribute to the mechanical attachment of trophozoites to both the intestinal wall and the glass surface tube in axenic culture. As a result, the cytoskeleton, particularly the ventral disk, plays important role to survive the trophozoites into the intestine (Elmendorf et al. 2003). There is no evidence of cellular invasion or receptor-mediated attachment, and attachment depends on active metabolism. The survival of *Giardia spp* in the host's intestine is highly dependent on the function of the cytoskeleton, particularly the ventral disk. The factor such as reduce cysteine concentration, high oxygen levels and below temperature 37°C can causes inhibition of trophozoites attachment (Feely et al. 1982 & Gillin et al. 1982).

2.5.3 Flagella

The number of four pairs of flagella of *Giardia* trophozoites originating from basal bodies located near the anteroventral and midline to the nucleus. The flagella emerge from the region of the anterior, posterior, caudal, and ventral sites of trophozoites. The paraflagellar rods have present in one ventral side of the trophpzoites (Feely et al. 1990; Holberton et al. 1973). The flagella are composed of nine pairs of microtubules encircling two microtubules, which are essential for motility and play a role in excystation, as they emerge early during the process of excystation (Buchel et al. 1987). Despite not being directly involved in attachment, flagella

play a vital role in the survival of parasites. Annexin XXI is a protein that is specifically localized in the flagella of *G. lamblia* (Szkodowska et al. 2002).

2.5.4 Median Body

The cytoskeleton of *Giardia* spp includes a distinctive structure called the median body and it is a distinct morphology in differentiating between different *Giardia* species. The median body *in G. lamblia* trophozoites is usually represented by two claw hammer-shaped microtubule bundles present in the dorsal and midline to the caudal flagella. Recent research has revealed specialized membranes in *G. lamblia* with electron transport and membrane-potential-generating capabilities (Lloyd et al. 2000). These findings provide further support to the emerging theory that *Giardia* may not be a primitive organism but rather evolved from an aerobic, mitochondria-containing flagellate.

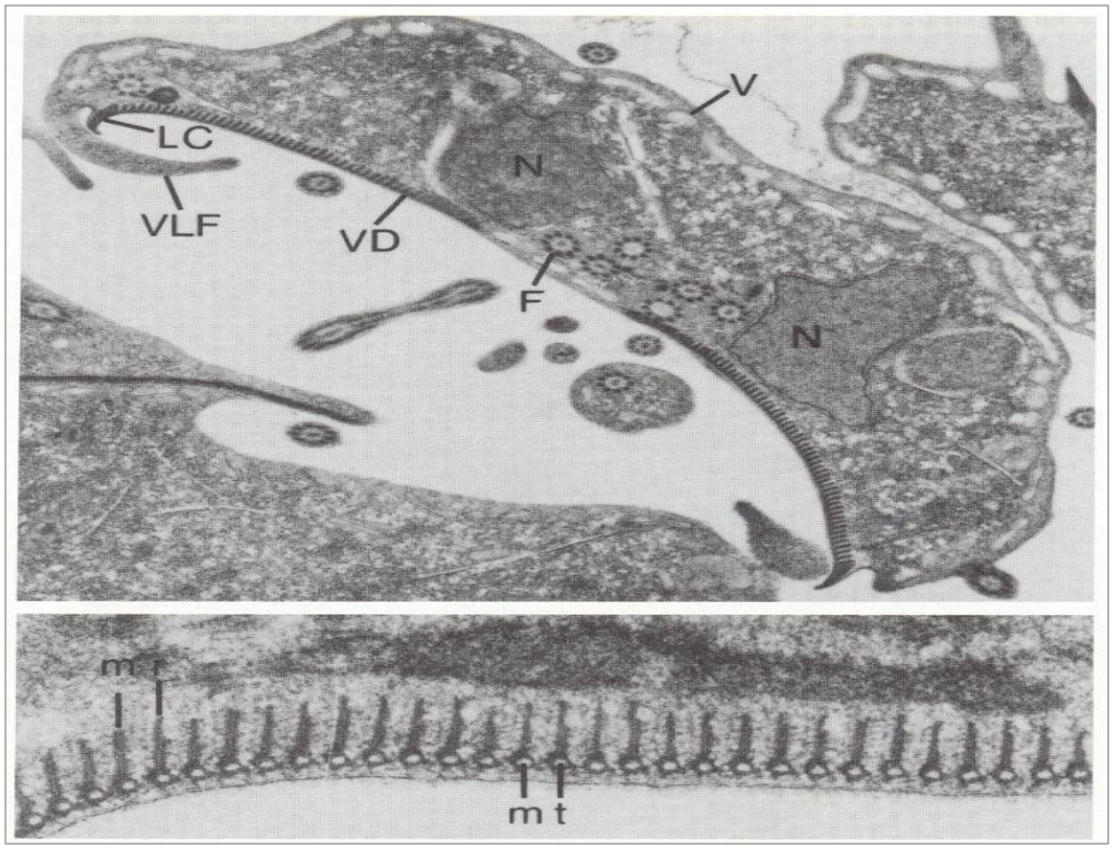


Figure 2.5: Cross sectional transmission electron micrograph of *G. lamblia* trophozoite. LC: lateral crest, VLF: ventrolateral flange, VD: ventral disk, N: nucleus, F: flagella, V: vacuole. In the lower panel, a higher magnification shows the microtubules (mt) and microribbons (mr).

2.5.5 Nuclear Structure and Replication

The trophozoites of *G. lamblia* possess two nuclei that are nearly indistinguishable in appearance. These nuclei undergo replication at approximately the same time (Wiesehahn et al. 1984) and furthermore, both nuclei are transcriptionally active, as confirmed by the incorporation of uridine into nuclear RNA. The two nuclei of *G. lamblia* are equally partitioned during cytokinesis (Yu et al. 2002). They contain rDNA amount same as showed by in situ habitation using the rDNA prob. Additionally both nuclei have same amount of DNA which is determined by nuclear staining with DAPI (Kabnick et al. 1990) or propidium iodide, (Bernander et al. 2001) although the equality of DNA content has been a topic of debate. It is widely accepted that the two nuclei of *G. lamblia* possess the same set of genes and chromosomes. This assumption is supported by research using fluorescence in situ hybridization with single-copy genes.

2.5.6 Mitosome

Giardia belongs to the group of diplomonads, which are often characterized as an ancient type of protist due to their lack of conventional eukaryotic organelles (such as mitochondria and peroxisomes), underdeveloped endomembrane system, and early divergence in various gene phylogenies (Riordan et al. 2003). The *Giardia* origin of putative mitochondria regarding nuclear gene (Roger et al. 2002) along with the discovery of mitochondrial remains in amitochondrial protists like *Entamoeba histolytica* (Koonin 2003; Gray 2005) and *Trachipleistophora hominis* (Gribaldo et al. 2002), imply that the absence of mitochondria in some eukaryotes is not a primitive state, but rather the outcome of evolutionary reduction. The presence of mitochondrial mitosomes has been confirmed in *Giardia* through the use of specific antibodies targeting IscS and IscU (Figure 2.6). These two proteins are involved in the biosynthesis of iron-sulfur clusters, and the mitosomes are surrounded by double membranes while serving a function in the maturation process of iron-sulfur proteins. Contrary to being considered a primitive amitochondrial organism (Tovar et al. 2003), *Giardia* has instead maintained a functional organelle originating from the original mitochondrial endosymbiont. These results suggest that *Giardia* has undergone evolutionary changes, including the retention of a modified mitochondrial organelle.

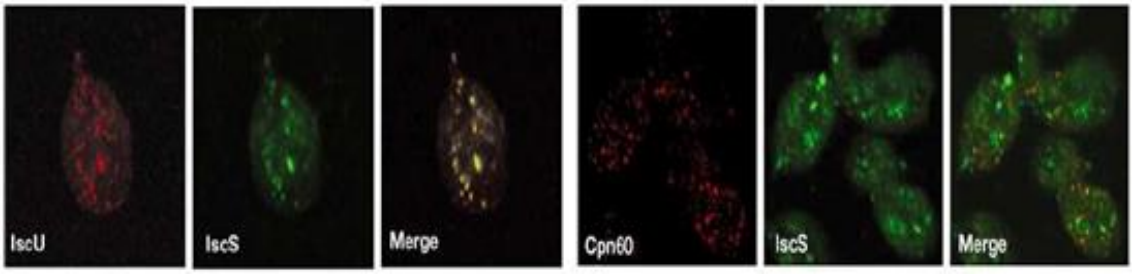


Figure 2.6: Evidence for presence of mitosome in *Giardia*

2.5.7 Cyst structure

Giardia cysts actually measure 5 by 7 to 10 µm are enclosed by a wall that is comprised of two membranes: an inner membranous layer and outer filamentous layer, with a thickness of about 0.3 to 0.5 µm. The major constituent of the cyst wall is a unique polysaccharide that contains N-acetylgalactosamine (GalNAc) and several proteins. The synthesis of GalNAc occurs through an inducible pathway of enzymes that convert fructose 6-phosphate to UDP-GalNAc during the encystment phase. UDP-GalNAc synthesis genes are activated at the transcriptional level during encystment, and GalNAc is not present during the growth phase of trophozoites (Lopez et al. 2003).

2.5.8 Cell Cycle

Based on flow cytometric analysis, the different stages of life cycle the DNA content (Bernander et al. 2001) proposed a model that illustrates the relationship between the life cycle of *Giardia* and its cell cycle. The proposed model suggests that during its life cycle, *Giardia* undergoes two phases of genome replication, which are followed by two phases of nuclear division, creating an alternating pattern between these processes. This cycle can be observed in early and late cysts, as well as in cells after excystment. *Giardia* trophozoites, which have two nuclei, follow a typical cell cycle where DNA replication occurs in the S phase, which involves cytokinesis and mitotic division. The S phase and M phases are segregated by gap periods (G1 and G2). This standard cell cycle allows the trophozoites to reproduce and propagate within the intestine as a pathogenic stage of the parasite. However, once the cells have finished replicating their DNA and entered the G2 phase, they undergo a differentiation process and transform into a cyst, which is the longest period of cell cycle of *Giardia* (Bernander et al. 2001; Hofstetrova et al. 2010). The beginning of the encystation process in *Giardia* is determined by a restriction

point, which present in G2 phase of the cell cycle. However, the specific factors that determine when a G2 cell is ready to undergo encystation are still unclear (Reiner et al. 2008). During the time of encystation, the two nuclei of trophozoites divide without undergoing cytokinesis, leading to the formation of four cyst nuclei, which is thought to be a variant of endomitosis. After the nuclei divide during encystation, the DNA content is duplicated. The result is a mature *Giardia* cyst that appears as a syncytium with four nuclei and a replicated genome (Bernander et al. 2001; Poxleitner et al. 2008; Erlandsen and Rasch 1994).

Except for *G. microti*, all other *Giardia* species have fully developed daughter individuals that are separated within the mature cyst (Feely 1988). In these species, cytokinesis occurs after excystment, which implies that there can be a considerable time gap between DNA replication and cytokinesis, lasting for several weeks or even longer, depending on the timing of cyst excystment. *Giardia* cysts need to be able to withstand a variety of external conditions, including those that can cause harm to their genome, such as exposure to UV light. Recent studies showed that cysts have the capacity to repair DNA damage caused by UV radiation (Li et al. 2008). The expression of putative meiosis genes such as DMC1A, SPO11, and HOP11 in cyst nuclei may be linked to this ability. These genes are responsible for DNA double-strand breaks and are thought to be involved in mitotic recombination of fused *Giardia* cyst nuclei during diplomyxis (Poxleitner et al. 2008). Although the destiny of a quadrinucleate cell during and after excystment is yet to be fully unclear, a hypothesis suggests that two rapid successive cell divisions take place based on combined FACS and microscopy data. This hypothesis proposes that these divisions result in the production of four trophozoites, each cyst contain a simple level of genome ploidy (Bernander et al. 2001).

2.5.8.1 Cell Division of *Giardia* Trophozoite

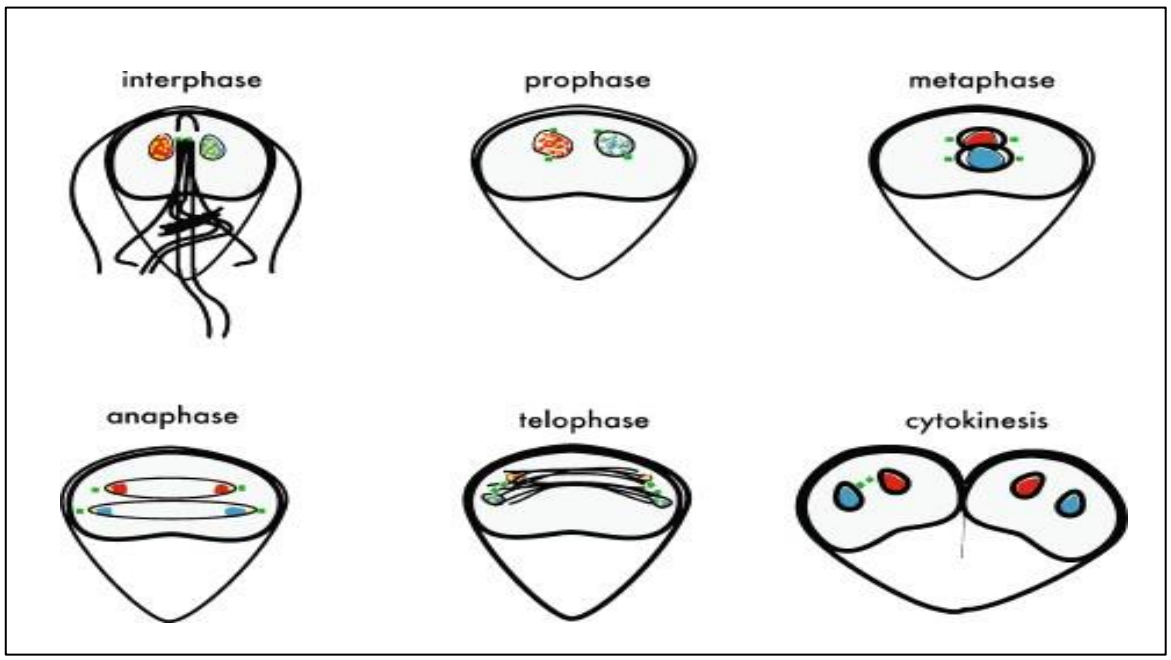


Figure 2.7: Schematic representation of cell division in *Giardia.*

Giardia is a unicellular organism consists of five chromosomes with a haploid genome size of 12 Mb, which ranging in size from 1.4 to 3.4 Mb (Adam 2000). The nucleus is considered diploid; the nuclei within trophozoites and cysts exhibit a diploid state (Bernander et al. 2001). *Giardia* undergoes asexual reproduction through binary fission, a process that has been widely recognized for more than half a century (Filice 1952). Several models concerning the plane of cytokinesis have been proposed in the past (Yu et al. 2002; Benchimol 2004). However, it is only recently that a comprehensive understanding of the cytokinesis mechanism has been achieved. This is due to a combination of new discoveries regarding the process of mitosis (Kulda & Nohynkova 1995), and the transformation of flagella, (Nohynkova et al. 2006). Additionally, studies on cytokinesis, (Tumova et al. 2016), have provided further insights that have helped clarify the true mechanism. To gain insights into the division process of *Giardia*, researchers used various techniques such as live cell monitoring and imaging methods including immunofluorescence, transmission, and scanning electron microscopy. The process of cell division involves the splitting of the anterior-posterior direction of parent cells and the resulting daughter cells separate from each other in a ventral-to-ventral orientation within the adhesive disk. This mechanism helps to maintain the *Giardia* left-right asymmetry (Kulda and Nohynkova 1995; Sagolla et al. 2006 & Tumova et al. 2007). The spiral layer of the disc

microtubules, which appears in a counter-clockwise direction when viewed from the ventral side, is one of the features that reflects this asymmetry. Variability in ploidy levels exists between the two nuclei in a single *Giardia* cell and among different strains belonging to distinct assemblages (Tumova et al. 2007). Furthermore, during division, *Giardia* undergoes alternating phases of attachment and free-swimming, which are determined by the adhesive competence of the parental or newly formed daughter discs and the specific phase of division. The process of *Giardia* division is typically divided into three distinct phases: the initial, terminal attachment phase and free-swimming phase. The initial attachment phase occurs within the adherent parent cell and involves several crucial processes related to the duplication of cell structures. During the initial attachment phase of *Giardia* cell division, the cell undergoes various key processes involved in the duplication of cell structures, such as reorganizing the parent flagella, assembling new flagella, undergoing mitosis, and disassembling the parent ventral disc. Additionally, during this phase, the cell initiates the assembly of new daughter discs. *Giardia* division is categorized into three phases: the initial, terminal attachment phase, and free-swimming phase (Tumova et al. 2007). During the initial phase, the cell undergoes several critical processes involved in duplicating cell structures, including cytokinesis initiated through the selection of the division site and the start of furrow ingression. The free-swimming phase continues this process of cytokinesis, and terminal phase is characterized by abscission, which physically separates the two daughter cells. The initial and free-swimming phases are relatively short, lasting around 3 and 1 minute, respectively, while the terminal phase can take up to 50 minutes, making it the longest phase of the division process. Although the cellular architecture of the phases of *Giardia* cell division is well-described, the molecular basis of these processes and the mechanisms regulating and controlling them remain to be fully elucidated.

Mitosis

Giardial mitosis has been investigated for more than 50 years, despite recent controversies (Felice 1952). Using cytological markers, a recent study has confirmed that the key cytological events during mitosis in *Giardia* adhere to a pattern similar to that observed in numerous other organisms. The process starts with chromatin condensation during prophase, followed by chromosome movement towards the spindle midzone in metaphase. Chromosomes then move towards the spindle poles during anaphase A, followed by spindle pole separation in anaphase B. Finally, flagella and the ventral disc are duplicated before cytokinesis. Both spindles are bipolar microtubule arrays that attach to chromosomes via kinetochores.

Mechanism of Chromosome Segregation and Mitosis

The classification of protist mitotic spindles is based on several cytological characteristics, the cytological features used to classify mitotic spindles in protists include the following: during mitosis the nuclear envelop which can be open, closed or semi open the spindle position in relation to the nuclear envelope, categorized as pleuromitosis or orthomitosis, the chromosomes position in relation to the spindle axis (Raikov 1994). In contrast, nuclear envelope of maximum plants and animals completely breaks down at the time of prophase, the microtubules to interact directly with chromatin. While most protists undergo closed mitosis and intact nuclear envelope (as observed in some fungi), *Giardia* undergoes a semi-open mitosis, which is a subtype of closed mitosis. During semi-open mitosis, kinetochore microtubules enter the nucleus through polar openings in the nuclear envelope.

Prophase

In mitosis during prophase stage of *Giardia* trophozoites, there are several key events that occur, including spindle nucleation, chromosome condensation, nuclear repositioning, and assembly (Cerva & Nohynkova, 1992; Sagolla et al. 2006). Specifically, extensive chromosome condensation and spindle assembly, and nuclear repositioning are hallmarks of this stage. In fact, one of the initial events that takes place during prophase in *Giardia* is the condensation of chromosomes. As a result of this process, individual chromosomes become distinguishable and can be observed as discrete structures within the cell. This is an important step in the overall process of cell division in *Giardia*. Accurate chromosome segregation during mitosis in eukaryote occur on centromeric chromatin the assembly of the kinetochore and the following addition of the kinetochore to spindle microtubules. In many eukaryotic cells, the formation of the mitotic kinetochore relies on the recruitment of motors, checkpoint proteins, and other structural components by centromeric foci (Van Hooser et al. 2001). This process is also likely to occur during giardial prophase, given that the centromeres are visibly present on the condensed chromosomes (Sagolla et al. 2006). The clear visualization of centromeric foci during giardial prophase, indicating that the assembly of the mitotic kinetochore may follow a similar mechanism in this organism. In *Giardia*, the process of spindle nucleation and assembly takes place soon after chromosome condensation. This is typically observed during the prophase stage of mitosis. During this stage, the nucleation of two spindles becomes apparent as microtubules (MTs) begin to form between the two nuclei located near the flagellar basal

bodies. As mitotic spindles begin to develop, their MTs extend around each nucleus. Over time, spindle microtubules continue to elongate during nuclear migration, until each spindle completely encompasses its respective nucleus by the end of prophase. The process of spindle nucleation in *Giardia* is typically mediated by microtubule organizing centres (MTOCs) (Figure 2.7).

Metaphase

After nuclear migration in prophase, microtubules form two complete bipolar spindles separately one dorsal and one ventral (**Fig. 2.7)**. These spindles have opposing poles that align with the left-right axis of the cell, and the chromatin is tightly clustered in each centre of the spindle axis. On the metaphase plate the alignment of centromeres, if it exists, is uncertain and may only occur briefly as a transitional stage during metaphase.

Anaphase A and B

The process of chromosome segregation in both nuclei can be divided into two stages: anaphase A, where chromatids are separated, followed by anaphase B, where spindle elongation takes place along the left-right axis (Fig. 2.7). One of the nuclei starts anaphase-A first, but anaphase B occurs simultaneously in both nuclei. The exact nucleus that initiates mitosis is not clear, and it is uncertain if the starting will occur in the same nucleus. At the time of anaphase, A, the centromeres migrate towards the spindle pole closest to the leading edge of the dividing DNA. This indicates that the kinetochore is connected to the microtubules for chromosome segregation. The separation of sister chromatids to opposite sides (L-R) during anaphase A is a result of both lateral chromosome segregation and migration of nuclear. Anaphase B is characterized by spindle elongation, which is observed in *Giardia* as the nuclei extend to the far L-R sides while the nuclear envelopes remain together. During anaphase B, centromeric foci stay closely at the spindle pole. Furthermore, in spindle pole the centrin foci stay throughout both anaphase A and B (Sagolla et al. 2006). A recent discovery revealed that in *Giardia*, a sole mitotic cyclin is necessary for the onset of mitosis. This is the first instance of mitosis not being controlled by the APC and it could be a manifestation of an ancient form of cell cycle regulation that has been retained throughout evolution (Gourguechon et al. 2013).

Telophase

Although the timing and process of mitosis may differ among various eukaryotes, the typical orientation of the plane of cell division is usually at a right angle to the axis along which the chromosomes are separated. This orientation is determined by the mitotic spindle (Balasubramanian et al. 2004). In *Giardia*, cytokinesis occurs in perpendicular to the spindle axis and this pattern of cytokinesis is in line with other eukaryotes. During telophase, a notable feature is the existence of a microtubule bundle that spans between the nuclei, with unfocused ends replacing the bipolar spindle arrays (Fig. 2.7). This structure may indicate that the spindle microtubules after the loss of spindle poles, or it could originate from the de novo polymerization of microtubules. Based on the localization of cenH3: GFP during telophase and the movement of centrin foci, it appears that the centromeres remain clustered and there is no nuclear fusion during mitosis in *Giardia*. As cytokinesis begins and the DNA decondenses, the centromeric foci become visible and the nuclei migrate to their respective positions in the daughter cells. Therefore, each of the two daughter cells inherits one copy of each parental nucleus in every generation.

Implications of mode of mitosis on nuclear inheritance and heterozygosity

In addition to defining the plane of cytokinesis, the manner in which *Giardia* undergoes cell division carries crucial implications for preserving a distinct genetic identity for each nucleus. It is widely believed that both nuclei possess identical genetic material (Kabnick & Peattie 1990; Yu et al. 2002). Notably, previous research has demonstrated that *Giardia* intestinalis assemblage A exhibits a minimal degree of genetic heterozygosity, which amounts to less than 0.1% (Baruch et al. 1996; Lu et al. 1998). Assemblage B, among others, has been found to exhibit higher levels of heterozygosity as compared to assemblage A (Franzen et al. 2009), while some have demonstrated same pattern of heterozygosity (Jerlstrom-Hultqvist et al. 2010). It has been suggested that the low levels of heterozygosity in *Giardia* could be explained by the both copies inherited either the left or right nucleus by a single daughter, in different generation which would effectively eliminate sequence (Yu et al. 2002). However, this explanation does not account for observed differences in ploidy (Tumova et al. 2007). On the other hand, the inheritance of one copy of both the left and right nuclei would maintain any genetic variations between the two nuclei in the daughter cells. Despite the absence of direct evidence for canonical meiosis, such as the presence of a synaptonemal complex, in trophozoites, the inheritance of genetic material from both nuclei may play a vital role in the survival of the cell.

Division of Microtubule Cytoskeleton

The process of cytoskeleton division is necessary for maintaining cell identity in the progeny (Nohynkova et al. 2006; Tumova et al. 2007). This division process is comparable to the mitotic division of nuclei. The cytoskeleton comprises the adhesive disc, eight flagella, and the funis, all of which must be duplicated. Prior to these studies, the division process was not well understood. However, the research showed that after extensive reorganization occur in interphase flagellar at the time of less than three minutes, converting all but the parent caudal flagella into different flagella in the progeny.

Division of the flagellar apparatus

Recently reviewed by Dawson and House (2010), the complex flagellar apparatus of *Giardia*, a diplomonad, is well understood in terms of its basic features. It consists of four different types of flagella: anterior, posteriolateral, ventral, and caudal. Each flagella type in the diplomonad cell architecture is composed of two bilaterally symmetric flagella and distinguished by its position, organization, subordinate structures, and probable functions.Two symmetric clusters of flagellar basal bodies, referred to as tetrads, are located between the anterior poles of the two nuclei in *Giardia*. These tetrads, together with two microtubular root fibers, specific flagella, and other fibrillar appendages, form a structural unit known as a mastigont, as described by Kulda and Nohynkova (1995). During cell division in *Giardia*, eight parent flagella remain present. In semi-conservative manner the parental flagella are passed down to the daughter cells, so that from parent cell four flagella received by each progeny, which are then combined with four newly generated flagella to create a perfect daughter set. Traditionally believed that each daughter cell of *Giardia* inherits the same set of parent flagella, (one mastigont). However, this understanding was recently challenged. The notion that the distribution of parent flagella to daughter cells in *Giardia* is uniform was primarily supported by evidence demonstrating that the cleavage plane during cytokinesis divides the dividing cell between separated mastigonts, as observed in previous studies (Filice, 1952; Cerva & Nohynkova, 1992). However, other models of cytokinesis did not follow the segregation of flagella or anticipated symmetric distribution.

Parent flagella distribution

Recent research has utilized monoclonal antibodies specific to acetylated and polyglycylated tubulins, alongside scanning and transmission electron microscopy, to shed new light on the process of cytokinesis in *Giardia*. This work has revealed that prior to cytokinesis, the two parent mastigonts engage in an exchange of half of their flagella components, as described by Nohynkova et al. 2006. These recent findings challenge the previously held notion that each daughter cell of *Giardia* receives one mastigont from each parent cell, instead revealing that half of each of the two mastigonts is received between each daughter cells. This is due to the arrangement of basal body pairs in tetrads, resulting in each daughter cell inheriting a different set of four parent flagella. Furthermore, this research has shown that the flagella of *Giardia* undergo a maturation process.

Transformation of parent flagella during division

Giardia division involves a balanced distribution of the flagella from the parent anterior and caudal pairs between the progeny, with each daughter cell receiving one flagellum from each pair (Figure 10.2). On the other hand, the flagella of the parent ventral and posteriolateral pairs are unevenly distributed, resulting in one daughter cell inheriting both ventral flagella while the other daughter cell receives both posteriolateral flagella. Unequal segregation during *Giardia* division results in each daughter cell inheriting a distinctive set of four parent flagella. In the progeny, the majority of parent flagella undergo extensive mitotic reorganization, except for the caudal ones. This reorganization is accomplished by reorienting, migrating, and segregating the paired basal bodies, which causes the corresponding flagella to change positions and transform into different flagella types. In each progeny, the parent anterior flagellum undergoes transformation into a daughter caudal flagellum, which involves exchanging positions of the basal bodies of the flagella. The basal bodies undergo reorientation and migration towards opposite sides of the *Giardia* cell, with the left basal body moving towards the right and the right basal body moving towards the left side to achieve this transformation. During basal body migration, the intracytoplasmic segments of the corresponding axonemes move gradually towards the interior of the *Giardia* cell. This movement leads to the separation of the anterior cross-section of the axonemes, which eventually leads to the exchange of the positions at which the flagella exit, relative to the interphase. After transformation, the flagellum joins a segregated parent caudal flagellum to create a pair of daughter caudal flagella.

De Novo assembly of daughter flagella

During mitosis, newly assembled basal bodies give rise to de novo daughter posteriolateral (PL) and ventral (V) flagella pairs. New basal body formed by triggering parent basal body, resulting in the interphase and progeny cells having one new basal body pair and one old (parental) and, arranged in the characteristic *Giardia* configuration.The process of *Giardia* basal body duplication appears to follow the conservative mechanism observed in other cells for centriole/basal body duplication (Loncarek & Khodjakov, 2009). Specifically, the formation of each new basal body occurs near an old basal body, suggesting that it arises through the duplication of an already-existing structure. In *Giardia*, the conservative mechanism of duplication of centriole/basal body involves the stringent orientation of the new basal body of parent organelle right angles despite the non-orthogonal arrangement of basal body pairs during interphase. The control of basal body orientation during duplication is a characteristic feature of centriole duplication, which is maintained by a canonical mechanism. This mechanism restricts the number of newly formed basal bodies to one per parent organelle and cell cycle, ensuring strict regulation of basal body duplication.Although the details of this restriction process are not fully understood, it appears to be conserved in *Giardia*, as well as in other cells (Loncarek & Khodjakov, 2009; Tsou & Stearns, 2006; Azimzadeh & Bornens, 2007).

Maturation of flagella

In *Giardia*, the transformation of parent flagella and the de novo assembly of daughter flagella result in flagella of varying ages in each progeny and interphase cell. The ventral and posteriolateral flagella are the youngest as they are newly formed during mitosis.

In *Giardia*, the anterior flagella are one cell cycle old and are formed through the transformation of either a ventral or a posteriolateral flagella pair. The right caudal flagellum is older, having been formed through the transformation of a previous anterior flagellum. The left caudal flagellum, which is the oldest of all the flagella within the cell, is at least three cell cycles old. This flagellum represents the final stage of *Giardia* flagellum development, corresponding to a mature flagellum.

Developmental asymmetry of microtubular roots of caudal flagella

The flagellar maturation process in *Giardia* results in a caudal pair of flagella that have different ages (the second oldest and oldest). This asymmetry is specific to the basal caudal bodies and flagella plays a crucial role in preserving the cell asymmetry of left right, which is shown by the presence of a ventral disc. The *Giardia* cytoskeleton includes a single-copy component that originates from a microtubular root of basal body. Diplomonads caudal basal body contains two-root fiber, but in *Giardia*, the anterior root fiber is developed asymmetrically, as reported by Brugerolle in 1991.

Ventral Disc

While persistent parent basal bodies serve as templates for new basal bodies, which are produced in a conservative manner, new daughter discs in *Giardia* are assembled independently from the parent structure. During the formation of new daughter discs, the parent structure disintegrates simultaneously, and the new disc is always assembled de novo as a modified microtubular root.

Cytokines

During the cell cycle, cytokinesis is the last stage that separates a single cell into two daughter cells (Fig. 2.7). While the process of cytokinesis differs among eukaryotes, such as the division site being regulate by the mitotic spindle, in budding yeast the buck neck, and the nucleus positioning in fission yeast. In eukaryotes, cytokinesis occurs perpendicular to the axis of segregated chromosomes. In animals and yeast, the division of the mother cell into two daughters is facilitated by the constriction of the actomyosin ring (Barr & Gruneberg, 2007). However, the plane of cytokinesis in *Giardia* has been a topic of controversy for several decades. Several incongruous models have been proposed regarding the plane of cytokinesis in *Giardia* (Kabnick & Peattie 1990; Ghosh et al. 2001; Solari et al. 2003). Some models were based on a single observation (Solari et al. 2003 & Benchimol 2004) while others were derived from theoretical observations, such as the single nucleus labeling of episomal DNA by FISH in interphase cells (Yu et al. in 2002). However, most of these models were unable to explain the duplication of other cell systems, such as the nuclei and flagellar apparatus.

In *Giardia*, the initiation of cytokinesis is coordinated with the deactivation of mitotic cyclin-dependent kinases. During the free-swimming phase of cell division, the cleavage furrow moves in the anterior-posterior direction between the caudal axonemes of daughter cells, leading to

the separation of the detached parent cell along its longitudinal body axis. Proper membrane biosynthesis is crucial for successful cytokinesis, particularly in this phase. Studies have indicated that the progression of furrowing can be halted by inhibiting the synthesis of sphingolipids, which are essential components of eukaryotic membranes (Sonda et al., 2008). In *Giardia*, the two daughter cells mechanically separate ventral-to-ventral, with a yin-yang symbol's two-fold rotational symmetry. The ventral discs in both daughters exhibit a counterclockwise winding (Tumova et al. 2007).

2.6 Genetics and Molecular Biology

2.6.1 Genome structure

G. lamblia genome displays typical eukaryotic features such as the presence of linear chromosomes with telomeres that share a sequence (TAGGG) with other eukaryotes. Like other eukaryotes, the chromosomal DNA of *G. lamblia* is organized into chromatin by binding to a linker histone (H1) and four core histones (H2a, H2b, H3, and H4) to form nucleosomes. The core histones are highly similar to those present in other eukaryotes and do not exhibit any significant similarity in Archaea the histone-like proteins and all four core histones are present in its genome (Wu et al. 2000). To identify size variants of chromosome 1 in *G. lamblia*, researchers initially used several chromosome-specific probes, which revealed that bands that appeared lighter when stained with ethidium bromide were indeed variants of chromosome 1 (Adam et al. 1988). Further mapping of these different size variants allowed for a more detailed understanding of their genomic characteristics.

Research on *G. lamblia* genome has revealed that chromosome 1 displays size variations that range from 1.1 to 1.9 Mb, with changes occurring in both subtelomeric regions. Studies have demonstrated that the ribosomal DNA (rDNA) unit, which is made up of tandem repeats, was situated near the telomeric repeat region (TAGGG). This rDNA unit often played a role in subtelomeric rearrangements (Adam et al. 1991; Le Blancq et al. 1991; Le Blancq et al. 1992). The rDNA repeat is located on various chromosomes, even among same genotype isolates (Adam 1992). For instance, in the ISR isolate, the rDNA repeats are present at one end of chromosome 1, and roughly, 30% of the difference in chromosome size between isolates is feature to differences in the number of rDNA copies (Adam 1992). The remaining size difference is believed to result from dissimilarities in repetitive DNA close to the other telomere. The genome size is around 12.3 Mb ((Adam et al. 1988; Fan et al. 1991) and it exhibits variations ranging from 10.6 to 11.9 Mb based on different analytical methods. Comprising five

distinct chromosomes, the genome features individual sizes of 3.8, 3.0, 2.3, 1.6 and 1.6 Mb. However, it should be noted that variations in chromosome structure have been observed in different strains of *G. lamblia*. The sizes of the chromosomes vary from 0.7 to over 3 Mb and the genome sequencing technique used is shotgun sequencing. The estimated genome size of approximately 12 Mb is consistent with data obtained from *G. lamblia* genome analysis (available at www.mbl.edu/*Giardia*). Sexual organisms typically have lower levels of heterozygosity due to meiotic recombination, but asexual organisms can have much higher levels of allelic heterozygosity (Welch et al. 2000). *Giardia* are considered ancient asexual organisms, and it might very high grade of allelic heterozygosity in their genomes.

Surprisingly, despite being an ancient asexual organism, it has been found to have a relatively low degree of allelic heterozygosity, and the underlying reason for this is not yet fully understood. It is possible that unrecognized sexual processes may occur in *Giardia* or that there are intermittent losses of a nucleus that contribute to the low levels of allelic heterozygosity observed.

2.6.2 Transfection

The progress in creating transient and stable-transfection systems has greatly aided our comprehension of the genetics of *G. lamblia*. The first transfection system was described as involving the temporary expression of luciferase. This was accomplished by placing the luciferase gene in between a short section of the GDH gene at the 5' end and the presumed polyadenylation signal at the 3' end. Electroporation was the method utilized to introduce DNA in both the initial and subsequent transfection systems. Later on, stable episomal transfection was conducted by incorporating either the puromycin-resistance pac gene or the neomycin resistance neo gene as selectable markers. The transfection vectors were built on bacterial plasmids, which contained sequences that served as origins of replication in *G. lamblia*.

2.6.3 Transcription and Translation

Transcription in *G. lamblia* retains some eukaryotic characteristics, it also exhibits several traits that are typical of prokaryotes. For example, it is amanitin-resistant and carried out by RNA polymerase II (Seshadri et al., 2003). The transcript is synthesized in the nucleus, similar to all eukaryotes, for translation transported to the cytoplasm. While the polyadenylation of transcripts is a typical feature of eukaryotes, the short 5' untranslated regions (UTRs) and general scarcity of introns in *G. lamblia* are more akin to prokaryotes, though infrequent introns

may be observed in unicellular eukaryotes. Additionally, unlike other eukaryotes, most transcripts in *G. lamblia* a 5' cap do not appear to possess. The short 5' UTRs also raised the possibility that *G. lamblia* promoters might be located in close proximity to the beginning of the open reading frames. A set of highly conserved motifs was observed in the 5'-flanking regions of several cytoskeletal genes provided support for the idea that *G. lamblia* promoters might be situated near the beginning of the open reading frames. Additionally, a bi-directional promoter that facilitated divergent transcription of a PHD-zinc finger protein gene and a ran gene has been documented in *Giardia* (Ong et al. 2002).

At the site of transcription initiation, a consensus sequence AATTAAAAA was identified, with the actual initiation site located at TA or CA. Additionally, a promoter region was proposed 20 to 35 nucleotides upstream from the transcription initiation site, with the sequence CAAAAA (A/T)(T/C)AGA(G/T)TC(C/T)GAA. Another consensus sequence, CAATTT, was found at 40 to 70 nucleotides upstream from the initiation site. Confirmation of the GDH promoter's location near the coding region and its short length was achieved through a transfection assay, where a 44-bp sequence of the GDH gene showed maximal transcriptional activity.

An investigation into the GDH promoter region utilizing deletion and mutational analysis revealed the significance of the AT-rich region at the initiation site and a CAAAT region 34 base pairs upstream (Yee et al. 2000). Moreover, the specific binding of a 68 kDa nuclear extract protein to the immediate 51-base pair upstream region via band shift analysis and UV cross-linking (Yee et al. 2000). Through deletion and mutational analysis, it was found that the region from -51 to -2 of the ran promoter exhibited maximal promoter activity, while regions located further upstream did not contribute to the promoter's function. The -51 to -20 region was identified as the most crucial component for the promoter's activity, as smaller portions of this region led to reduced promoter activity. To investigate whether transcripts in *G. lamblia* possess 7-methylguanosine or other caps at their 5' ends, researchers analyzed the total polyadenylated RNA. The 5' ends were resistant to 5' phosphorylation, unless the RNA was pretreated with calf intestinal alkaline phosphatase to remove the 5' phosphates (Yu et al. 1998)). This indicates that *G. lamblia* transcripts lack 5' phosphates, a characteristic typically associated with capped RNA. The findings indicated that the RNA was phosphorylated but not capped, and the absence of an increase in phosphorylation after treatment with the decapping enzyme tobacco acid pyrophosphatase further supported the lack of 5' capping. As a result, it appears that the majority of polyadenylated RNA in *G. lamblia* does not possess a 7-methylguanosine cap. Although the studies were performed on total polyadenylated RNA, it cannot be ruled out that individual transcripts may have a 7-methylguanosine cap. In the case

of the glucosamine-6-phosphate isomerase B gene, differential processing of the transcript resulted in two variants with different 5' UTR lengths. The constitutive transcript with a short 5' UTR was found to be uncapped, whereas the transcript expressed during encystation with a longer 5' UTR (146 nucleotides) was found to be capped, as evidenced by RNA ligation after tobacco acid pyrophosphatase (Knodler et al. 1999) treatment. It was not determined whether this was a cap with a 7-methylguanosine or another type of cap.

Small nuclear RNA molecules, such as snRNA U1, U2, U4, U5, and U6, play a role in splicing nuclear pre-mRNA, while sno-RNA U3, U8, U14, snR10, and snR30 are involved in pre-rRNA processing, ultimately contributing to the generation of mature RNA (Niu et al. 1994). In many other eukaryotes, these molecules possess a trimethylguanosine cap at the 5' end. Anti-trimethylguanosine antiserum was used to immunoprecipitate a group of potential snRNAs from *G. lamblia*, and the presence of caps was confirmed by testing the antibody reactivity's sensitivity to the decapping effect of tobacco acid pyrophosphatase. However, the specific identities and functions of these candidate snRNAs have yet to be established. It would be particularly significant to investigate whether mRNA splicing occurs in *G. lamblia*, given that introns have not been detected in *G. lamblia* genes. However, a recent proposal by Nixon et al. 2003 suggests the presence of a spliceosomal intron in *Giardia*, making this topic even more intriguing for further research.

In the initial investigation of rRNA and rDNA genes in *G. lamblia*, it was discovered that the size of the rRNA molecules was significantly smaller than the typical eukaryotic 18S (SS rRNA) or 28S (LS rRNA) molecules. Surprisingly, these rRNA molecules were even smaller than the 16S and 23S rRNA molecules found in E. coli.The rDNA repeats in *G. lamblia* were also notably small, yet their organization and sequence were similar to those found in other eukaryotes. Moreover, other components that make up the translation machinery, such as elongation factor 1, elongation factor 2, RPB1, eRF1, eRF3, and RNA polymerase III, were distinctively eukaryotic in *G. lamblia.*

2.6.4 Transposons

Mobile genetic elements have a significant role to influencing the evolutionary trajectory of the genome due to their capability to relocate to novel chromosomal sites. These elements are prevalent in various organisms across the biological kingdom. Protozoan parasites exhibit several forms of transposable elements, with the most extensive diversity observed in the trypanosomatids. The identification of three families of transposon elements in the WB isolate

of *G. lamblia* that is presently utilized for the genome-sequencing project (Arkhipova et al. 2001). Of these, two are located in subtelomeric regions, while the remaining one is situated internally on a chromosome and is nonfunctional. According to Bhattacharya et al. 2002, a nucleotide sequence analysis of all the identified elements confirmed that they are retrotransposons, and none of them belongs to the non-long-terminal-repeat retrotransposon category. Moreover, these elements exhibit a distinct genetic makeup that sets them apart from all other known non-LTR lineages. The phylogenetic analysis suggests that the Genie elements (Early non-LTR insertion element) represent one of the earliest non-LTR element lineages that has undergone strict vertical descent (Burke et al. 2002). Despite accumulating mutations over time, many copies of these elements retain the potential to encode various activities such as endonuclease reverse transcriptase, and nucleic-acid-binding (Bhattacharya et al. 2002).

2.7 Protein Transport and Degradation

2.7.1 Endomembrane protein transport system

Although electron microscopy (EM) had previously identified structures that resembled endoplasmic reticulum (ER) in *G. lamblia*, the existence of ER was still uncertain. Recently SR characterization, cloning and the detection of a significant labeled of membrane system with an antibody (Soltys et al. 1996) have put these doubts to rest. These findings provide conclusive evidence for the presence of ER in *G. lamblia*. Standard microscopic techniques have failed to detect Golgi complexes in trophozoites, although they have been observed in encysting organisms, (Reiner et al. 1990). However, a more recent study (Lanfredi-Rangel et al. 1999) involving the freeze-fracture and transmission electron microscopy of nitrobenzoxadiazole (NBD) ceramide-labeled in trophozoites log phase has revealed perinuclear region staininng. This staining pattern as like as Golgi complexes from other organisms, thus indicating the presence of Golgi-like structures in *G. lamblia*. Despite the detection of Golgi-like structures in trophozoites through recent electron microscopy studies, additional research is necessary to validate their existence and to establish their features (Lujan et al. 2003). Additionally, for the specific transport of proteins into the nuclei, green fluorescent protein (GFP) can be directed specifically to the nuclei by utilizing the nuclear localization signal of simian virus 40 (Elmendorf et al. 2001). Many transporters located in the endoplasmic reticulum, plasma membrane, and acidocalcisomes work in tandem to regulate the cellular metabolism and distribution of calcium ions (Mineno et al. 2003).

Endosomes and lysosomes are present in the majority of eukaryotic cells to break down and recycle proteins that originate within the cell or that are taken up from the extracellular environment through processes such as endocytosis or phagocytosis. When endocytosed proteins enter the cell, they are initially transported to early endosomes, which can either mature into late endosomes or facilitate the protein's return to the cell membrane. From there, late endosomes carry proteins to lysosomes, where they are degraded. Endosomes and lysosomes are characterized by their acidic environments, with endosomes having a pH below 6 and lysosomes having a pH of 5. Trophozoites exhibit multiple vacuoles that surround the cell perimeter and possess certain characteristics of lysosomes and endosomes. These vacuoles demonstrate acidity, as demonstrated by their ability to absorb acridine orange (Feely et al. 1991; Kattenbach et al. 1991). Studies have shown that the vacuoles present in trophozoites have the ability to accumulate exogenous ferritin and lucifer yellow, indicating that they may play a role in the process of endocytosis (Lanfredi-Rangel et al. 1998; Tai et al. 1993). *G. lamblia* virus particles are gathered in these vacuoles via an endocytic mechanism. Pulse-chase labeling experiments with horseradish peroxidase have revealed that vacuoles are consistently labeled over time, suggests that unlike higher eukaryotes, trophozoites do not have a clear differentiation endocytic vesicles between early and late.

Lanfredi-Rangel et al. 1998 used zinc iodide-osmium tetroxide and glucose-6-phosphatase to label a subset of vacuoles in trophozoites, and performed three-dimensional reconstructions to investigate their connection with the endoplasmic reticulum (ER). Furthermore, Electron microscopy was employed in conjunction with anti-BiP antibody to substantiate the hypothesis that the vacuoles in trophozoites are linked to the endoplasmic reticulum (ER) (Soltys et al. 1996). In addition to their endocytic and ER-related functions, vacuoles in trophozoites also possess several hydrolase activities, including acid phosphatase, proteinase, and RNase, which are indicative of lysosomal properties. As a result, these vacuoles have been observed to perform the roles of both early and late endosomes, as well as lysosomes, and may be functionally linked to the ER (Lanfredi-Rangel et al. 1998).

2.8 Cell Biochemistry

G. lamblia is an anaerobic organism that is tolerant of oxygen, and unlike most eukaryotes, it does not have mitochondria. However, it possesses lysosome-like organelles. Until 1980, there was limited knowledge regarding the biochemistry and metabolism of *Giardia*. However, this situation changed significantly with the successful axenisation of trophozoite in vitro. Since

this breakthrough, numerous studies on the biochemical and metabolic characteristics of this organism have been published.

2.8.1. Carbohydrate Metabolism

Aerobic metabolism and cytochrome-mediated oxidative phosphorylation are the primary means of energy production for most eukaryotes. However, there are some eukaryotic organisms, such as *Giardia* spp. *Trichomonas* spp. *Entamoeba* spp. that do not possess mitochondria and rely on alternative pathways for energy production. *Giardia* spp., *Trichomonas* spp., *Entamoeba* spp. and produce energy fermentative metabolism process primarily, even when oxygen is present. Their metabolism is characterized by glycolysis and its limited extensions, which produce ATP only through substrate-level phosphorylation. Unlike aerobic metabolism, glucose is not fully oxidized to CO2 and H2O. Instead, it is incompletely catabolized into ethanol, acetate, alanine, and CO2. The production of these ends products is influenced by the oxygen tension and glucose concentration in the medium. Minor variations in oxygen levels have a significant impact on the metabolism of trophozoites. In the absence of oxygen, trophozoites primarily produce alanine from carbohydrate metabolism (Edwards et al.1992; Paget et al. 1990 &1993). However, the addition of small amounts of oxygen, even at concentrations as low as <0.25 µM, stimulates ethanol production and inhibits the production of alanine (Paget et al. in 1993). As oxygen concentrations increase further, both ethanol and alanine production are suppressed. When oxygen concentrations exceed 46 µM, the production of alanine is entirely suppressed, and the trophozoites primarily produce acetate and CO2 as the main byproducts of energy metabolism. These findings suggest that oxygen concentrations within the range of 0 to 60 µM, which are typical of the intestinal environment where the trophozoites reproduce, can have a significant impact on their metabolism. Specifically, at oxygen levels above 46 µM, the production of alanine is completely halted, and the trophozoites shift to producing acetate and CO2 instead. It appears that the metabolic pathway of pyruvate is modified in response to changes in the surrounding environment, such as varying levels of oxygen. Unlike in other organisms, *Giardia* lacks metabolic compartmentalization, and all reactions take place either in the cytosol or on the cytosolic surfaces of membranes, including the PFOR-mediated reactions (Lindmark 1980).

Glucose is the primary source of energy obtained from carbohydrates (Jarroll et al. 1995). The conversion of glucose to pyruvate occurs through both the hexose monophosphate shunt and Embden-Meyerhof-Parnas pathways (Figure 2.8). In most eukaryotic and prokaryotic

organisms, the transformation of fructose-6-phosphate to fructose-1, 6-bisphosphate is a regulated and irreversible step that relies on the activity of ATP-dependent phosphofructokinase. For *Giardia*, as well as for *T. vaginalis* and *E. histolytica,* the catalysis of this reaction involves a pyrophosphate-dependent phosphofructokinase. In contrast to its ATP-dependent counterpart, this enzyme facilitates a reversible reaction and operates without regulation (Mertens, 1993). The cloning and characterization of the pyrophosphate-dependent phosphofructokinase from *Giardia* have been demonstrated (Phillips et al. 1995; Rozario et al. 1995). Two enzymes responsible for converting phosphoenolpyruvate into pyruvate have been identified: ATP-dependent pyruvate kinase (Park et al. 1997) and pyrophosphate-dependent pyruvate phosphate dikinase (PPDK) (Bruderer et al. 1996; Hiltpold et al. 1999). The coordinated reaction involving adenylate kinase and PPDK in PPDK-mediated reactions can generate two molecules of ATP, which represents a potential energy advantage over ATP-dependent pyruvate kinase. Adenylate kinase has been identified, and it catalyzes a reaction that converts two ADP molecules into ATP and ADP in an essentially energy-neutral manner (Rozario et al. 1995). PPDK, on the other hand, the conversion of phosphoenolpyruvate to pyruvate, PPDK converts phosphoenolpyruvate and AMP into pyruvate and ATP, ultimately resulting in the net production of two ATP molecules. This represents an energy advantage over the pyruvate kinase reaction, which produces only one ATP molecule. Although their relative roles in glycolysis are still unknown, the higher specific activity of pyruvate kinase suggests that it might have a more significant role in the process compared to PPDK (Park et al., 1997). The pyruvate converted to acetyl-CoA is facilitated by PFOR in organisms like *E. histolytica*, which uses ferredoxin instead of NAD as the electron acceptor (Lindmark 1980; Townson et al. 1994 & 1996). This is in contrast to aerobic eubacteria and eukaryotes, where the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase complex. In *E. histolytica*, acetyl-CoA can follow two pathways: it may be converted to acetate through the action of ADP-forming acetyl-CoA synthetase, which generates ATP from ADP during the reaction (Sánchez et al. 2000). Alternatively, acetyl-CoA can be transformed into ethanol by the bifunctional enzyme alcohol dehydrogenase E. This enzyme features acetaldehyde dehydrogenase activity at its amino terminus, facilitating the conversion of acetyl-CoA to acetaldehyde, while its carboxy terminus hosts alcohol dehydrogenase activity, leading to the conversion of acetaldehyde to ethanol (Dan et al. 2000; Sánchez 1996&2000). The bifunctional enzyme alcohol dehydrogenase E possesses two distinct activities: an acetaldehyde dehydrogenase activity located at its amino terminus that catalyzes the conversion of acetyl-CoA to acetaldehyde, and an alcohol dehydrogenase activity located at its carboxy terminus that converts the acetaldehyde

to ethanol.

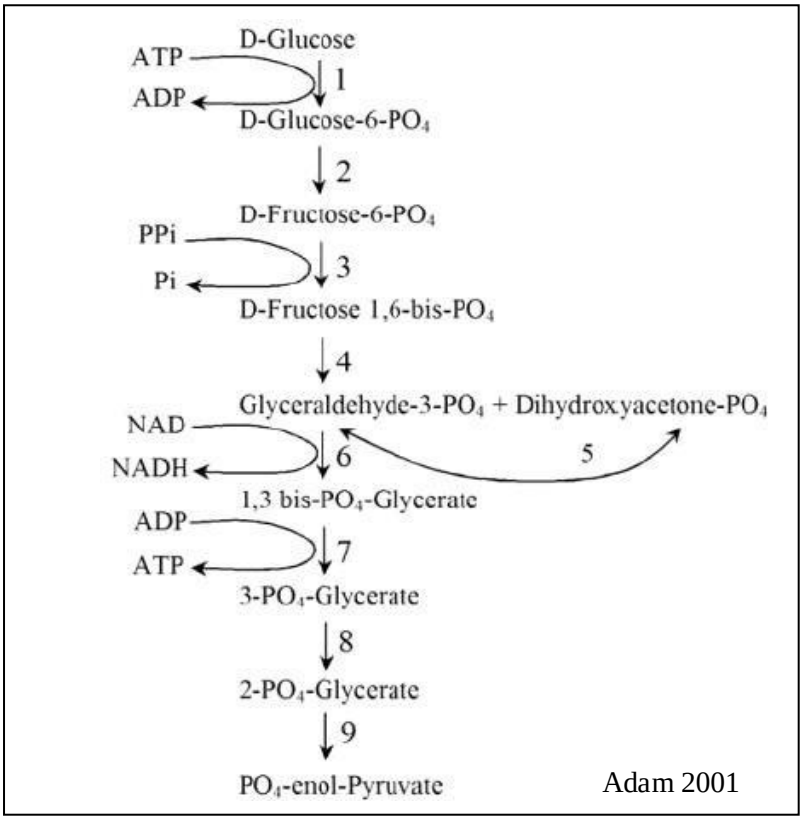


Figure 2.8: Metabolism of glucose to phosphoenolpyruvate. The enzymes are labeled as follows: 1 - hexokinase; 2 - glucose phosphate isomerase (proposed); 3 - pyrophosphate-dependent phosphofructokinase; 4 - fructose bisphosphate aldolase; 5 - triosephosphate isomerase; 6 - glyceraldehyde-3-phosphate dehydrogenase; 7 - phosphoglycerate kinase (proposed); 8 - phosphoglyceromutase (proposed); 9 - enolase (proposed).

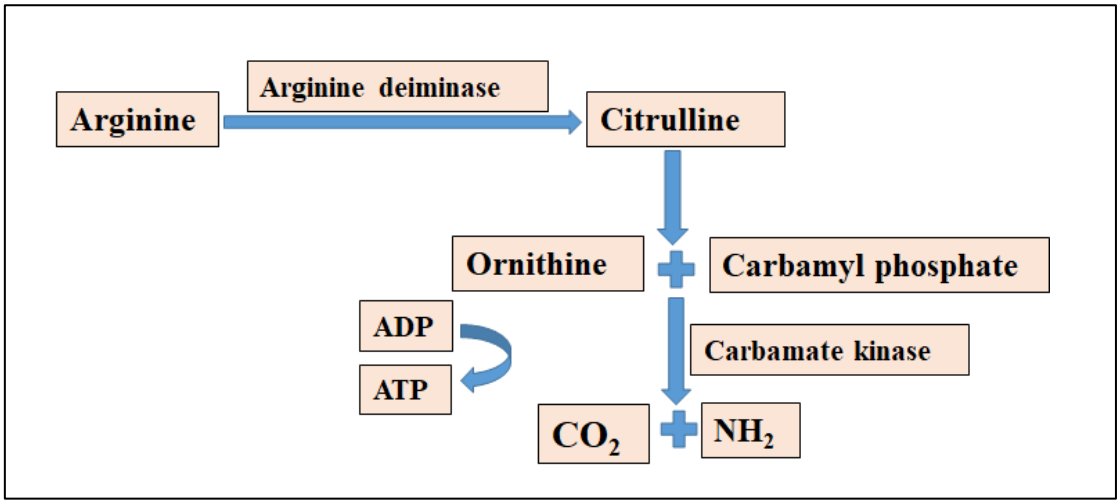


Figure 2.9: Arginine dihydrolase pathway**:** The enzymes are labeled as follows: 1- arginine deiminase; 2 - ornithine transcarbamoylase; 3 - carbamate kinase.

A breakthrough has been made in understanding the core sugar metabolism of *Giardia* through the application of conventional bioinformatic methods. By comparing the proteomes of *Giardia* with enzymes already known from other organisms, researchers have successfully identified enzymes involved in crucial metabolic pathways. Specifically, enzymes related to glycolysis, as well as certain enzymes associated with the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, have been discovered in *Giardia*. This finding provides valuable insights into the sugar metabolism of *Giardia* and enhances our understanding of its cellular processes. The recent identification of *Giardia's* core sugar metabolism using bioinformatic approaches revealed an intriguing finding. While enzymes in the glycolysis pathway were successfully identified, most enzymes associated with the tricarboxylic acid (TCA) cycle and oxidative phosphorylation were not detectable, suggesting the potential absence of these functionalities in *Giardia*. Interestingly, the enzymes found in *Giardia*'s glycolysis pathway exhibit greater similarity to those found in bacteria (Han et al. 2012). Furthermore, the reconstruction of various sugar-related metabolic pathways in *Giardia* has shed light on significant enzyme absences within these pathways. These discoveries provide valuable insights into the unique metabolic characteristics of *Giardia* and highlight distinctions in its sugar metabolism compared to other organisms. By employing bioinformatic techniques, researchers have made an intriguing observation regarding *Giardia*'s glycolytic enzymes. These enzymes exhibit a closer resemblance to their bacterial counterparts, as opposed to enzymes found in eukaryotes or archaea. Notably, phosphoglucomutase and phosphoglycerate kinase in *Giardia* bear a higher similarity to those found in eukaryotes. However, the identification of only a limited number of enzymes from the tricarboxylic acid (TCA) cycle and oxidative phosphorylation strongly suggests the probable absence of these pathways in *Giardia*. This finding underscores the unique characteristics of *Giardia*'s sugar metabolism and indicates significant differences when compared to other organisms. The methodology of analyzing metabolic pathways described above holds potential for application to any organism possessing genome information but limited annotation. One key advantage of this approach is its relatively rapid nature, providing initial insights into the likely presence or absence of specific pathways. However, it is important to acknowledge certain limitations. In some instances, the allocation of incorrect EC numbers by KEGG for a small number of proteins can lead to false positives. This highlights the necessity for users to possess a familiarity with the pathways being examined in order to accurately interpret the results. Nonetheless, despite these limitations, this approach represents a valuable tool for gaining preliminary understanding of an organism's potential metabolic pathways based on available genomic data.

In addition to the aforementioned limitations, it is important to note that false positives can arise when dealing with EC groups that exhibit significant similarity to each other. For instance, confusion may arise between enzymes such as succinyl-CoA synthetase and acetyl-CoA synthetase, leading to potential false positives in the analysis. It is also crucial to acknowledge that while KEGG is a valuable database, it is still expanding and not all enzymes from every species have been included in its repository. Therefore, researchers should exercise caution and consider these factors when utilizing KEGG or similar databases for pathway analysis, recognizing the evolving nature of such resources. It is important to consider that certain enzyme candidates might not be identified if they originate from a species that is highly distinct from those with known enzymes. Taking the overall metabolic profile of *Giardia* into account, it appears that glucose is taken up from the host and metabolized via glycolysis, leading to the production of pyruvate. Subsequently, to regenerate the oxidized form of coenzyme NAD+, pyruvate is further metabolized through reduction into ethanol, alanine, or acetate. The specific end product formed depends on the availability of oxygen within the environment. This metabolic pathway provides a comprehensive understanding of how glucose is utilized by *Giardia* and highlights the mechanisms it employs to maintain its energy metabolism and redox balance. In the presence of oxygen (aerobic conditions), pyruvate in *Giardia* is converted to alanine through a transamination reaction or to acetate via the action of acetyl-CoA synthetase.

However, under anaerobic conditions, pyruvate is metabolized to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (PFOR). Subsequently, acetyl-CoA is further transformed into acetaldehyde and ultimately ethanol. It is noteworthy that the tricarboxylic acid (TCA) cycle and oxidative phosphorylation pathways do not appear to be operative in *Giardia*. These observations provide important insights into the metabolic adaptations of *Giardia* to different oxygen conditions, indicating alternative pathways for energy production and redox balance maintenance. The discovery of the absence of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation pathways in *Giardia* aligns with previous expectations, considering the organism's anaerobic lifestyle. *Giardia* is recognized for its adaptation to an anaerobic environment, a process that has led to genome reduction and the development of a smaller genome containing fewer nonessential enzymes. This streamlined genome provides the parasite with an advantage during replication. Furthermore, *Giardia* shares several metabolic characteristics with bacteria, including its reliance on fermentative energy metabolism. Notably, *Giardia* predominantly utilizes pyrophosphate (PPi) rather than adenosine triphosphate (ATP) for its energy requirements. These findings further highlight the unique

evolutionary adaptations of *Giardia* and its resemblance to bacterial metabolic processes.

Morrison et al. 2007 conducted a study that offered a brief insight into the metabolic repertoire of *Giardia* upon the completion of the *Giardia* genome project. Their findings revealed a distinctive combination of sugar metabolic pathways in *Giardia*, consisting of a mixture of enzymes that exhibited similarities to both eukaryotic and bacterial enzymes. Specifically, some enzymes appeared to be more closely related in sequence to enzymes found in eukaryotes, while others showed a stronger resemblance to bacterial enzymes. This unique blend of eukaryote-like and bacteria-like enzymes in *Giardia*'s sugar metabolic pathways underscores the organism's distinct evolutionary history and highlights its intriguing metabolic adaptations. In 2007, Morrison et al. provided valuable insights into the composition of *Giardia*'s glycolytic enzymes, noting that approximately half of these enzymes exhibited similarities to eukaryotic enzymes. However, it is important to clarify that the study did not distinguish between the enzymes commonly studied in well-characterized eukaryotes such as mammals, yeasts, and plants, and enzymes specifically found in eukaryotic protists. The study specifically treated protists as a distinct group within eukaryotes because protists often possess enzymes that more closely resemble those found in prokaryotes rather than the enzymes observed in the extensively studied eukaryotes. This differentiation highlights the unique enzyme profile of eukaryotic protists, including *Giardia* and further emphasizes their evolutionary divergence from other well-studied eukaryotes. The presence of bacteria-like enzymes in *Giardia* can be explained by mitochondrial genes migrating to the nucleus, lateral gene transfer events involving bacterial genes, and the evolution of a unique set of enzymes after the divergence of *Giardia* from its ancestral eukaryotic form. These factors collectively contribute to the notable presence of bacteria-like enzymes in *Giardia*'s metabolic repertoire.

The glycolysis pathway is highly conserved among organisms, with minor variations. However, the conservation of enzymes in the glycolytic pathway across all organisms is not absolute, as there can be some variability in specific enzyme isoforms or adaptations to suit different biological contexts. Nonetheless, the core enzymes involved in glycolysis are generally present and functionally conserved across a wide range of organisms. In a comparative analysis of *Giardia*'s annotated proteins (total 4889), they were compared against 28 bacterial, 12 archaeal, and 17 other eukaryotic species. This analysis resulted in the identification of four distinct groups of proteins based on their conservation across the three domains of life. Group A consists of 37 *Giardia* proteins that are conserved in all three domains of life, namely bacteria, archaea, and eukaryotes. Group B comprises 849 *Giardia* proteins that are found in all eukaryotes, indicating their widespread conservation across eukaryotic organisms. Group C includes 274

eukaryotic signature proteins (Hartman et al. 1941; Han et al. 2012), which are conserved in all eukaryotes but are not found in archaea or bacteria. These proteins represent a distinctive set of eukaryotic markers. Lastly, Group D consists of 278 proteins from Escherichia coli that are conserved in all bacterial species, illustrating their conservation within the bacterial domain. This comprehensive analysis provides valuable insights into the conservation and distribution of proteins in *Giardia* across different domains of life, serving as a foundation for further studies on the evolutionary relationships and functional characteristics of these proteins. Out of the 20 candidate glycolytic enzymes, none were found to match with Group A (conserved in all three domains), Group C (eukaryotic signature proteins), or Group D (conserved in all bacterial species). However, six of the candidate enzymes were identified as matches within Group B, which represents proteins found in all eukaryotes. This indicates that these particular glycolytic enzymes are conserved across a wide range of eukaryotic organisms. These findings highlight the significance of studying the functional conservation and evolutionary implications of glycolysis within the context of eukaryotic metabolism.

The findings suggest that *Giardia* exhibits a combination of eukaryote-like and prokaryotic-like enzymes in its glycolytic pathway. Additionally, glycolysis in bacteria shows considerable diversity in its forms. Consequently, none of the bacteria-like glycolytic enzymes found in *Giardia* are expected to be universally present in all bacteria. As a result, their matches with proteins in Group A or Group D are less likely. These observations underscore the unique nature of *Giardia*'s glycolytic enzyme repertoire and highlight the need for further investigation into the evolutionary aspects and functional significance of these enzymes in the context of the parasite's metabolism. The glycolytic enzymes in eukaryotes exhibit higher conservation across different eukaryotic organisms. Consequently, some of *Giardia*'s eukaryote-like glycolytic enzymes were identified as conserved in all eukaryotes. However, it should be noted that homologues of these enzymes, which are conserved across eukaryotes, could also be found in certain branches of bacteria. This is due to the wide range of glycolytic enzyme variations present in bacteria. As a result, these enzymes did not appear in Group C, which represents eukaryotic signature proteins.

It is important to recognize that the current findings serve as an initial indication and will serve as a foundation for future studies. Further investigations will delve deeper into understanding the significance of these conserved glycolytic enzymes in different organisms and their evolutionary implications. By employing a similar method, additional pathways such as amino acid metabolism and the RNA degradation pathway can be investigated, contributing further insights into *Giardia*'s metabolism. Furthermore, this study successfully identified potential

Giardia enzymes that were previously unrecognized. These newly discovered candidates demonstrate significant similarity to known enzymes within their respective classes. While their precise functions are yet to be confirmed, these findings provide valuable guidance for future experimental confirmation through activity assays. This ongoing research holds the promise of unraveling the intricate details of *Giardia*'s metabolic processes and expanding our understanding of this fascinating organism. A drug target is often a crucial molecule that plays a significant role in the infectivity or survival of a microbial pathogen. Achieving selective toxicity is most effective when the parasite possesses a key enzyme that either differs significantly from those found in the host or is absent in humans. For instance, *Giardia* harbors the enzyme PFOR, whereas the host (such as humans or mammals) utilizes the pyruvate dehydrogenase complex for a similar function. This disparity has enabled the design of drugs, like metronidazole, that specifically target PFOR in *Giardia*. Such targeted drugs take advantage of the divergent enzymes or pathways in the parasite, aiming to disrupt their vital processes and combat the infection while minimizing harm to the host. In the context of the glycolytic pathway, certain enzymes in *Giardia*, such as glucokinase and phosphofructokinase, exhibit significant differences compared to their counterparts in the host. Glucokinase, which has been studied as a potential drug target for type-2 diabetes, presents an uncertain potential as a target for parasite infection. On the other hand, phosphofructokinase has been proposed as a drug target for *E. histolytica* (Byington et al. in 1997). In their study, they designed a competitive inhibitor specifically targeting phosphofructokinase, which successfully inhibited the growth of the parasite in vitro. These findings suggest the potential of phosphofructokinase as a viable target for therapeutic interventions against certain parasites, offering possibilities for further exploration and development of anti-parasitic drugs. Enzymes like glucokinase and phosphofructokinase, particularly those that can be substituted by alternative pathways in the host, present promising prospects for the development of new drug targets against *Giardia*. By gaining a deeper comprehension of this parasite's metabolism, we can unlock additional opportunities to combat this global parasitic challenge. Enhancing our understanding of *Giardia*'s intricate metabolic processes will undoubtedly equip us with more effective tools to address this widespread issue and advance the development of targeted therapeutic intervention.

2.8.2 Amino Acid Metabolism

Recent studies have highlighted the growing significance of amino acids in the energy metabolism of *G. lamblia*. The absorption of alanine aspartate and arginine from the surrounding environment, coupled with evidence of glucose-independent metabolism, indicates the potential significance metabolism of of amino acid in generating energy within *Giardia* (Mendis et al. 1992; Schofield et al. 1990 & 1991) (Fig. 2.6). The arginine dihydrolase pathway, documented in *T. vaginalis* (Lindmark et al. 1982) and *G. lamblia*, represents a potential energy source (Edwards et al. 1992; Schofield et al. 1990 & 1992). While this pathway is commonly found in prokaryotic organisms, its presence among eukaryotes is limited. Within the arginine dihydrolase pathway, arginine undergoes conversion into ornithine and ammonia, resulting in ATP generation through substrate-level phosphorylation (Fig. 2.9). Subsequently, the export of ornithine occurs through a transporter mechanism that exchanges it for extracellular arginine (Schofield et al. 1995). In addition, aspartate represents another potential energy source for *G. lamblia*. Through the action of aspartate transaminase, aspartate is converted to oxaloacetate, which enters the intermediary pathway. Within this pathway, oxaloacetate undergoes conversion to pyruvate, with a malate intermediate (Mendis et al., 1992). Under isoosmolar conditions, the intracellular concentration of alanine in *G. lamblia* is measured to be 50 mM (Knodler et al. 1994; Park et al. 1997). However, when exposed to hypoosmolar challenges, the concentration of alanine decreases rapidly due to an active transport mechanism. Moreover, apart from alanine, potassium has been observed to play a role in the osmoregulation of *G. lamblia* trophozoites (Maroulis et al. 2000). The secretion of alanine is facilitated by a specialized transporter that also transports glycine, L-serine, L-glutamine, L-threonine, and L-asparagine (Edwards et al. 1993). This transporter functions as an antiport system, exchanging intracellular alanine for these other amino acids from the extracellular environment (Schofield et al. 1995).

Giardia relies on scavenging amino acids from its intestinal environment as it lacks the ability to synthesize most amino acids, except for alanine and valine, which are produced as by-products of energy metabolism. An essential requirement for the axenic growth of *G. lamblia* trophozoites is a relatively high concentration of cysteine (16 mM). Interestingly, cysteine provides partial protection against oxygen toxicity, unlike other reducing agents such as cystine. The specific protective effect of cysteine has been demonstrated (Gillin et al. 1981 & 1982). *G. lamblia* does not produce cysteine de novo or synthesize it from cystine (Lujan et al. 1994). Cysteine is thought to enter the *G. lamblia* cell primarily through passive diffusion, although

active transport mechanisms may also contribute (Lujan et al. 1994). The importance of free thiol groups on the trophozoite surface was demonstrated by the toxicity of thiol-blocking agents, which failed to penetrate intact cells (Gillin et al. 1984). The observed toxicity suggests that these agents react with thiol groups on the trophozoite surfaces, leading to their death. Cysteine has been identified as the primary thiol group present in *G. lamblia* (Brown et al. 1993). Notably, *G. lamblia* relies on cysteine as its primary low-molecular-mass thiol, and the presence of glutathione (GSH) has been reported to be absent. However, the *G. lamblia* genome contains putative genes for the enzymes involved in GSH biosynthesis, such as γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2) (Morrison et al. 2007). Recent studies have unveiled the existence of a monothiol glutaredoxin (1-C-Grx) in *G. lamblia* (Rada et al. 2009). These small redox proteins are characterized by their CGFS active site motif and have the capacity to form dimers by coordinating a [2Fe2S] cluster. The active site cysteines of each monomer, along with the cysteines of two bound glutathione (GSH) molecules, contribute to this coordination. The primary role of 1-C-Grxs is believed to be associated with the biosynthesis of mitochondrial iron-sulfur clusters, likely representing the final step in this process. Specifically, the 1-C-Grx protein in *G. lamblia* is localized in the mitosomes, organelles similar to mitochondria that are involved in iron-sulfur cluster biosynthesis. Experimental evidence using recombinant *G. lamblia* 1-C-Grx has demonstrated the formation of a homodimeric complex incorporating iron-sulfur clusters, which can be stabilized by glutathione (GSH) (Rada et al. 2009). These findings suggest that *G. lamblia* may possess relatively low levels of GSH, which may have a specific role in the biogenesis of iron-sulfur clusters. Moreover, studies employing metabolic labeling with radiolabeled cysteine have indicated that the majority of the label is integrated into variant-specific surface proteins (VSPs) in *G. lamblia* trophozoites (Adam et al. 1988; Aggarwal et al. 1989). This observation suggests that these surface proteins may play a protective role in shielding the trophozoites from oxygen toxicity.

2.8.3 Lipid metabolism

The initial indication of the potential significance of bile in the proliferation of *Giardia* trophozoites was observed through their predominant growth in the jejunum and duodenum. In the absence of serum, bile can provide short-term axenic support for the growth of these trophozoites. Additionally, the growth can also be sustained by specific components of bile, such as cholesterol, phosphatidylcholine, glycocholate, and glycodeoxycholate, which are

biliary lipids and bile salts, respectively (Gillin et al. 1986). For extended axenic growth, serum is necessary. However, studies have demonstrated that the Cohn IV-1 fraction of bovine serum, which is increase in lipoproteins, alpha globulins and growth factors, can serve as a substitute for whole serum (Lujan et al. 1994). Notably, insulin-like growth factor II, found within the IV-1 fraction, stimulates the growth of *Giardia* trophozoites and enhances their uptake of cysteine. Similar effectiveness in supporting growth was observed when using the IV-1 fraction obtained from several other mammalian species, although in some cases, antibody depletion was necessary (Lujan et al., 1994).

G. lamblia trophozoites lack the ability to synthesize fatty acids from scratch, except for a few specific minor fatty acids (Ellis et al. 1996). However, the presence of free fatty acids is detrimental to the trophozoites. Instead, these organisms seem to meet their lipid needs by acquiring cholesterol and phosphatidylcholine from their external environment (Lujan et al. 1996). The necessary phospholipids and cholesterol for *Giardia* trophozoites are provided through, cyclodextrins, lipoproteins and bile salts, with the assistance of bile salts facilitating the lipids transfer to the parasite surface. Additionally, there is a possibility of a limited degree of lipid uptake through endocytosis. Exogenous phospholipids have been observed to undergo fatty acid remodeling through deacylation/reacylation reactions, enabling the alteration of lipids without the need for synthesizing entirely new phospholipid molecules (Das et al. 2002). The uptake of conjugated bile acids by *Giardia* trophozoites seems to occur through a carrier-mediated mechanism, involving distinct carriers for cholylglycine and cholyltaurine (Das et al. 1997). The primary fatty acids detected in trophozoites grown in axenic culture are stearic acid, palmitic acid, and oleic acid. Furthermore, the presence of fatty acid desaturase activity has been observed, including the desaturation of oleate to linolenate and linoleate (Ellis et al. 1996). Neutral lipids, phospholipids, and various cellular lipids in *Giardia* trophozoites incorporate arachidonic acid (Gibson et al. 1999). On the other hand, palmitic acid, oleic acid and myristic acid are primarily transformed into phospholipids, including important cellular phospholipids such as phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (Mohareb et al. 1991; Stevens et al. 1997). In addition, interesterification takes place involving the inclusion of conjugated fatty acids into phosphatidylglycerol. The detrimental effects of specific analogs of phosphatidylglycerol on *Giardia* trophozoites have been observed, although the precise mechanism behind this toxicity remains undetermined (Gibson et al. 1999). Isoprenoids are a class of lipids derived from mevalonate and are commonly present in eukaryotic cells. While cholesterol is the most well-known end product

of isoprenoid synthesis, these lipids are also incorporated into proteins through posttranslational modification, particularly in the case of GTP-binding proteins. The process of protein isoprenylation has been confirmed through the inclusion of radiolabeled mevalonate into proteins of *Giardia* trophozoites (Lujan et al. 1995). The incorporation of mevalonate and cell growth were both reversibly hindered by competitive inhibitors targeting 3-hydroxy-3-methylglutoryl (HMG)-CoA reductase, an enzyme involved in an early step of isoprenoid synthesis. In contrast, inhibitors targeting later stages of isoprenylation irreversibly impeded cell growth.

2.8.4 Purine and pyrimidine salvage

Pathogenic protozoa, including *G. lamblia*, have been shown to rely on salvage pathways for acquiring purine nucleosides (Wang et al. 1983). Additionally, studies have demonstrated that *G. lamblia* (Aldritt et al. 1983), *Tritrichomonas* foetus (Wang et al. 1983) and *T. vaginalis* (Wang et al. 1984) lack the ability to synthesize pyrimidines and instead depend on salvage pathways to obtain both purine and pyrimidine.

Radiolabeled precursor studies have revealed that adenine, adenosine, guanine, and guanosine are incorporated into nucleotides during purine metabolism. However, in de novo synthetic pathway the component such as glycine, formate, inosine, hypoxanthine, or xanthine, were not observed to be incorporated. This suggests that the purine nucleosides, specifically adenosine and guanosine, are most likely imported into the cells by a transporter that exhibits broad specificity for both nucleosides and deoxyribonucleosides (Baum et al. 1993; Davey et al. 1992). Following that, the purine nucleosides undergo degradation to their respective bases facilitated by specific hydrolases. Phosphoribosyl 1-pyrophosphate, synthesized through the action of phosphoribosyl 1-pyrophosphate synthetase (Kyradji et al. 1998), then engages in a reaction with the salvaged purine bases. This reaction, catalyzed by the corresponding monophosphate phosphoribosyltransferase (PRTase), leads to the production of nucleoside-5'-monophosphate (Fig. 2.10). While GPRTases from most eukaryotes typically utilize hypoxanthine or xanthine as substrates, the GPRTase in *G. lamblia* exhibits a high specificity for guanine (Sommer et al. 1996). This specificity strongly suggests that guanine serves as the exclusive source of guanine nucleotides in *G. lamblia*. Metabolite analyses have provided evidence of specific depletion in the guanine ribonucleotide pools, which correlates with slower cell growth. These findings highlight the crucial role of guanine in supplying the necessary nucleotides for the growth and multiplication of *Giardia* (Munagala et al. 2002). According to

Sommer et al. 1996, GPRTase enzyme and it's the amino acid sequence in exhibits less than 20% identity with the human enzyme. The GPRTase crystallographic structure of has shed light on potential explanations for the distinctive substrate specificity observed in this enzyme. The presence of an aspartate substitution for leucine at position 181 could contribute to the unique substrate recognition of the *G. lamblia* enzyme (Adam 2001). Purine phosphoribosyltransferases are enzymes that facilitate the Mg2+-dependent reaction, converting a purine base into its corresponding nucleotide (Sarver et al. 2002). Specifically, *G. lamblia* adenine phosphoribosyltransferase (APRTase) exhibits nucleophilic displacement through electrophile migration (Shi et al. 2018). Furthermore, *G. lamblia* trophozoites rely on salvaging exogenous thymidine, uridine and cytidine for the synthesis of pyrimidine nucleotides, as depicted in Figure 2.11.

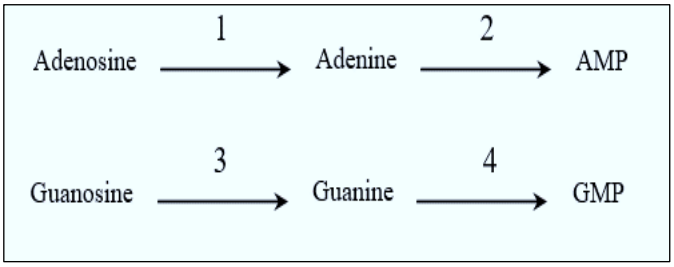


Figure 2.10: Purine ribonucleosides salvage pathways. The enzymes are labeled as follows: 1 - adenosine hydrolase; 2 - adenine phosphoribosyltransferase (APRTase); 3 - guanosine hydrolase; 4 - guanine phosphoribosyltransferase (GPRTase).

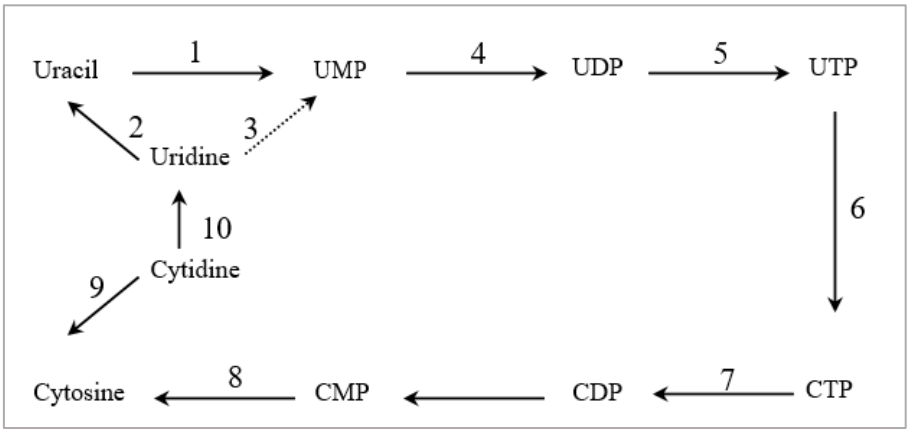


Figure 2.11: Pyrimidine ribonucleoside salvage pathway in *Giardia*. The enzymes are labeled as follows: 1 - uracil phosphoribosyltransferase (UPRTase);2 - uridine/thymine phosphorylase; 3 - uridine phosphotransferase (kinase); 4 - UMP kinase; 5 - UDP kinase; 6 - CTP synthetase; 7 - CDP kinase; 8 - cytosine phosphoribosyltransferase (CPRTase); 9 - cytidine hydrolase; 10, cytidine deaminase.

2.9. Immunology

In recent years, there has been notable advancement in comprehending the influence of the host immune system on the course of *Giardia* infection. The observation of natural clearance of infection in both humans and animals provides compelling evidence for the presence of a defensive immune response against *Giardia*. The higher occurrence of giardiasis in hypogammaglobulinemic patients highlights the potential significance of antibodies in providing immunity against this parasite. Numerous outbreaks of giardiasis have revealed that individuals with repeated exposure to *G. lamblia* have a reduced risk of infection, indicating that previous exposure confers partial resistance to reinfection. Furthermore, the elevated prevalence of *Giardia* infection among homosexuals (Brown et al., 1975; Hurwitz & Owen, 1978) indicates the potential involvement of cellular immunity of infection, particularly due to the presence of T-cell defects in many homosexual individuals. Studies have demonstrated impaired elimination of *Giardia* muris in mice with hypothalamic dysfunction as well as in mice lacking B-cells and mast cells, suggesting their contribution to immunity against this parasite. Additionally, macrophages have been implicated in the immune response against *Giardia*. It is evident that multiple immunological mechanisms play a role in protecting against *Giardia* infection.

2.9.1 Humoral response

In patients with *giardia*sis and malabsorption (Ridley & Ridley 1976) were the first to demonstrate the presence of serum antibodies to *G. lamblia* using the indirect immunofluorescence antibody test. These antibodies, including IgG, IgM, and IgA, were observed to target surface antigens of the parasite and the process of antibody-mediated killing may or may not involve complements (Deguchi et al. 1987; Nash & Aggarwal 1986).

The fact that antibodies are found in high titres in certain patients with recurrent infection, while absent in others, suggests that antibodies alone may not provide sufficient protection. In addition to antibodies, a cell-mediated response may be necessary to mount an effective immune response (Eckmann et al. 2003). Consequently, a combination of various immunological processes is required to successfully eliminate the *Giardia* infection, leading to the development of immune memory in most affected individuals. However, the specific mechanisms by which secretory antibodies and cellular components interact with *Giardia* remain largely unknown. Several researchers have explored the potential involvement of specific immunoglobulins in conferring resistance to giardiasis. It has been hypothesized that a

deficiency of these immunoglobulins could allow for the growth of the *Giardia* organism. For instance, the concentration of IgA in intestinal secretions was lower in giardiasis patients compared to individuals in control groups (Zinneman & Kaplen 1972)). In contrast, Char et al. 1993 found that children with persistent giardiasis exhibited elevated levels of total serum IgG, IgM, and IgA concentrations compared to control subjects. Analysis of *Giardia*-specific antibody concentrations indicated that only IgM levels were elevated, while the concentrations of IgA and IgG were comparable to those of the control group. The presence of a high concentration of *Giardia*-specific IgM, coupled with low concentrations of *Giardia*-specific IgG and IgA and the inability to successfully clear the infection, suggests an inefficient transition from an IgM response to an IgG or IgA response.

2.9.2 Cell mediated response

The understanding of the cell-mediated immune response in giardiasis remains limited; however, the involvement of T lymphocytes in this process has established. Evidence indicates that in cases of giardiasis, the total number of T lymphocytes in Peyer's patches increases twofold. Additionally, studies using nude mice (athymic mice) have demonstrated the development of chronic giardiasis, further emphasizing the role of T lymphocytes in the immune response to *Giardia* infection.

Multiple studies (Owen et al. 1981 & 1979) have shown that in vitro experiments using scanning microscopy techniques, macrophages derived from both infected and noninfected hosts have the ability to phagocytose *Giardia* trophozoites. These studies have observed that when trophozoites manage to breach the mucosal layer, macrophages extend pseudopodia through the epithelial basement membrane to engulf the trophozoites. This phenomenon highlights the ability of macrophages to capture and internalize *Giardia* trophozoites that have entered the mucosal tissues. Based on the existing evidence, it is suggested that macrophages do not possess the ability to spontaneously eliminate *Giardia* species. However, they serve important functions such as acting as antigen-presenting cells for CD4 lymphocytes and potentially participating killing of trophozoites in antibody-mediated process. These findings indicate that macrophages play important role in the immune response against *Giardia* infection by presenting antigens to CD4 lymphocytes and potentially contributing to the elimination of trophozoites through antibody-mediated mechanisms.

In theory, antitrophozoite antibodies could potentially provide a protective effect by inhibiting the attachment of *Giardia* trophozoites to intestinal epithelial cells or by opsonizing

the trophozoites for ingestion or killing by phagocytes. Evidence supports the notion that attachment of trophozoites is inhibited by antibody to the intestinal epithelium. This suggests that antibodies may play a role in preventing the initial attachment of *Giardia* trophozoites to the intestinal cells, potentially hindering their ability to establish infection. In addition to the immune protection system, nonimmune factors contribute to the defense against *Giardia* infection. These include the intestinal motility, mucosal layer, and the presence of human breast milk in infants (Adam 1991; Tellez et al. 2003). Furthermore, a T-cell-dependent mechanism has been identified in the control of acute *Giardia* infection, operating independently of antibodies and B cells (Singer & Nash 2000). Additionally, the significant role of IL-6 in the control of acute giardiasis has been studied (Bienz et al. 2003; Zhou et al. 2003).

2.9.3 Antigenic variation

2.9.3.1 Occurrence of Antigenic variation

Giardia trophozoites exhibit antigenic variation in their cysteine-rich surface antigens (Adam et al. 1988), a phenomenon observed within a family of immunodominant antigens in both laboratory settings (in vitro) and living organisms (in vivo). The initial investigations of *G. lamblia* surface antigens revealed variations among strains using techniques such as crossed immunoelectrophoresis and ELISA. Furthermore, significant differences were observed in the excretory-secretory products mass derived from various surface-iodinated *G. lamblia* isolates. In a study involving 19 isolates, it was found that the surface antigens of *G. lamblia* exhibited variability in both the number and size, ranging approximately from 50 to 200 kDa (Nash et al. 1985). Among these antigens, a monoclonal antibody known as MAb 6E7 specifically targeted a 170-kDa surface antigen initially referred to as CRP170 but now recognized as VSPA6, derived from the WB isolate. Notably, this monoclonal antibody demonstrated cytotoxic effects on WB trophozoites but did not exhibit similar effects on other surface antigens (Nash et al. 1986). Subsequent data has provided confirmation that individual *Giardia* organisms express a single variant surface protein (VSP) at any given time (Nash et al. 1990). The presence of multiple surface-labeled bands observed in certain studies can be attributed to the existence of subpopulations of trophozoites expressing various types of VSPs. According to Nash et al. 1990, in axenic culture, variation in *Giardia* occurs at an approximate frequency of 10^3 to 10^4, taking place once every 6 to 12 generations. Additionally, during acute giardiasis, several proteins including, alpha-giardins, variable surface proteins, arginine deiminase, fructose-1,6-bisphosphate aldolase and ornithine carbamoyl transferase, have been identified as

immunoreactive. The identification of these proteins has significant implications in advancing the development of novel diagnostic tools and vaccines for the detection and prevention of giardiasis (Palm et al. 2003).

2.9.3.2 Other antigens

The surface antigen of *G. lamblia*, Taglin, displays a migration pattern as a doublet with bands at 28 and 30 kDa on Western blots (Ward et al. 1987). Additionally, it has been reported to possess lectin activity that is induced by protease (Lev et al. 1986). Following trypsin treatment, Taglin demonstrates binding affinity towards mannose-6-phosphate. Notably, this antigen remains nonvariable, with its expression remaining constant throughout the processes of excystation and encystation (Svärd et al. 1998). Alpha-1 giardin is a GAG-binding protein with significant immunoreactivity, potentially serving a crucial function in mediating the interaction between the parasite and its host (Weiland et al. 2003)). *G. lamblia* trophozoites express a 49-kDa antigen known as GP49, which is anchored to the membrane surface through a glycosylphosphatidylinositol (GPI) attachment (Das et al. 1991). The synthesis of the GPI anchor entails the assimilation of external phosphatidylinositol, with myo-inositol undergoing conversion to phosphatidylinositol before its integration (Subramanian et al. 2000). Unlike various other GPI-anchored surface molecules, such as the trypanosome variant surface glycoprotein, the GPI anchor linked to GP49 demonstrates resistance to cleavage by phospholipase C, (Das et al. 1991). Furthermore, GP49 remains constant across different isolates of *G. lamblia* and does not exhibit variation within individual isolates.

2.10 Pathology and pathogenesis

The precise mechanisms through which *Giardia* causes the disease are not yet fully understood. However, accumulating evidence suggests that the interplay between host's and the parasite the characteristics may influence the outcome of the infection. In *Giardia*, symptomatic cases the trophozoites utilize their ventral disc for attachment and can either create a barrier effect or cause brush border damage. Consequently, this can lead to a reduction in brush border enzymes, resulting in the malabsorption of fat, lactose, Vitamin B-12 and protein. Hypogammaglobulinemic patients may experience more pronounced pathological changes, which could explain the higher incidence of symptomatic giardiasis in these individuals (Lebwohl et al. 2003).

In a study involving gerbils and human volunteers, two isolates with distinct antigenic characteristics were utilized, revealing a significant disparity in their pathogenicity. Subsequent investigations have revealed that the same *Giardia* isolate exhibits differential antigen expression depending on the host. An example of this variability can be seen in a group of human volunteers, where the *Giardia* parasite expressed a 72 KDa surface antigen, leading to a successful infection. In contrast, in another group, the parasite exhibited 200 KDa surface antigens and did not cause any pathogenic effects. Although the available data is inconclusive, this characteristic of the parasite holds potential importance (Nash et al. 1990). The development of *G. lamblia* infection can be influenced by various host-related factors, including age, nutritional status, genetic predisposition (as indicated by a higher frequency of infection in blood group A), and different states of immunodeficiency. Additionally, it has been observed that *G. lamblia* infection is associated with adult reactive arthritis. The susceptibility to *Giardia* infection is determined by varying degrees of virulence, and host-related factors. Moreover, these differences in both the infecting strains and the host contribute to the diverse range of clinical presentations observed in giardiasis.

2.11 Epidemiology

Giardiasis significantly contributes to the global burden of waterborne diarrheal diseases, ranking second only to respiratory infections in terms of mortality and morbidity (Wolfe 1992). The infection is particularly prevalent in tropical and subtropical regions, with higher rates observed among socio-economically disadvantaged populations (Nimri 1994; Mukhtar 1994; Rajeswari et al. 1994; Yassin et al. 1999). The transmission of giardiasis is facilitated by unsanitary conditions, especially in areas with limited access to clean drinking water and proper sewage disposal systems, leading to fecal contamination of water sources. While in developing countries, the disease is endemic and can cause localized outbreaks in the United States due to contaminated drinking water. In India, intestinal parasites pose a significant health concern (Jindal et al. 1995; Saha et al. 1996). *G. lamblia* is recognized as the primary infectious agent in both adults and children, responsible for a significant number of infections (Singh et al. 2018; Sethi et al. 1999). The main mode of transmission for *G. lamblia* is through direct fecal-oral route. While foodborne transmission is less frequent, it has been well documented in various studies (Osterholm et al. 1981; Petersen et al. 1988).The occurrence of these outbreaks is frequently associated with the contamination of freshly prepared food by food handlers who are infected with the parasite.

The global population of immunosuppressed individuals is on the rise, driven not only by the ongoing HIV pandemic but also by factors such as malnutrition, cancer chemotherapy, and immunosuppressive therapy. This increase in immunosuppressed individuals puts them at a heightened risk of opportunistic parasite infections (Stark et al. 2009). In HIV-infected patients, giardiasis is not recognized as a significant contributor to enteritis, and it is not included in the list of opportunistic parasitic infections. This is because giardiasis typically does not cause long-term symptoms, and its treatment is not affected by the patient's immune status. The reported prevalence of giardiasis in HIV patients varies between 1.5% and 17.7%, (Stark et al. 2009). Symptoms of giardiasis in individuals with HIV infection show similarity and comparable severity to those in individuals without HIV infection. Asymptomatic giardiasis is frequently observed in HIV-positive individuals (Faubert 2000). However, when there is a decline in CD4+ cell counts leading to progressive immunosuppression, the risk of symptomatic infections increase, often presenting as chronic diarrhea (Dwivedi et al. 2007).

In the past, it was commonly believed that species belonging to the *Giardia* genus were eukaryotic organisms that lacked sexual reproduction in their uncomplicated life cycles (Adam 2001). However, several recent studies have questioned this perspective (Caccio & Sprong 2009). The exploration of the presence of recombination in *Giardia* has attracted attention not only from an evolutionary biology standpoint (Logsdon 2008), but also due to its significant implications for taxonomy, population genetics and the epidemiology of *Giardia*. Increasing evidence supports the occurrence of genetic exchanges within assemblages, including exchanges at the individual level or between individuals of the same assemblage and even between different assemblages. Genetic exchanges have been documented not just among human field isolates of assemblage A, subgroup AII (Cooper et al. 2007), but also among axenic isolates of subgroups AI and assemblages A and B (Teodorovic et al. 2007). Moreover, comparative and phylogenetic analyses of sequences from GenBank have provided evidence of genetic exchanges occurring among different *G. lamblia* assemblages (Lasek-Nesselquist et al. 2010).The differentiation between mixed infections and authentic recombinants is undeniably crucial. To establish this distinction conclusively, it will be necessary to conduct analyses at the level of individual cells, such as single cysts. Fortunately, the technical feasibility of genotyping single cysts has been demonstrated (Miller & Sterling 2007), and there are available PCR-based assays that are specific to different assemblages (Geurden et al. 2009; Almeida et al. 2010). Therefore, conducting research in this direction holds immense significance and should be prioritized. For inter- and intra-assemblage recombination to take place, it is necessary that

mixed *Giardia* infections occur within individual hosts. This phenomenon appears to be observed, particularly in dogs and humans (Sprong et al. 2009), where approximately 20% and 30% isolates, respectively, were found to be mixtures of inter-assemblage in MLG analysis. The presence of multiple *Giardia* genotypes carries important implications for comprehending the etiology of giardiasis. However, the exact mechanisms and timing of how humans and animals acquire infections with two or more genotypes remain uncertain. Different genotypes infection can occur through two possible scenarios. Firstly, it can happen simultaneously due to environmental mixing, such as in water sources. Alternatively, individuals may be infected asymptomatically with one assemblage but develop symptoms upon a second infection with another assemblage. The latter hypothesis gains support from the discovery of asymptomatic individuals. The presence of mixed infections holds significance for molecular typing of *Giardia*, enabling a better understanding of the genetic diversity and transmission patterns of the parasite. Relying solely on a single marker for assigning isolates to specific (sub)-assemblages may not always yield reliable results, as different markers can produce different outcomes. For example, one marker may classify an isolate as potentially zoonotic, while another marker may classify it as host-adapted. To obtain more reliable results, it is advisable to utilize multiple markers for typing purposes. By incorporating multiple markers, the accuracy and consistency of the typing process are improved, leading to a more comprehensive and robust characterization of the isolates. By utilizing multiple markers, the accuracy and consistency of the typing process are enhanced, leading to a more comprehensive understanding of the zoonotic potential and host specificity of the isolates.

2.12 Clinical features

The clinical presentation of giardiasis can range from no noticeable symptoms to persistent diarrhea accompanied by malabsorption. In individuals experiencing symptomatic giardiasis, the most common manifestation is diarrhoea characterized by foul-smelling stool, loose that may appear frothy, greasy or bulky. Additionally, patients often report gastrointestinal discomfort such as abdominal cramps and bloating, as well as symptoms like nausea and a reduced appetite. Most individuals affected by giardiasis commonly exhibit symptoms such as malaise and weight loss, and in some cases, fever can be present, especially during the initial phase of the infection (Moore et al. 1969). However, it is noteworthy that about half of those infected do not manifest any noticeable symptoms, and the infection frequently resolves spontaneously without the need for medical intervention (Adam 1991).

2.13 Diagnosis

Diagnosis of *G. lamblia* can be done in several ways (Kapoor *et al.*, 2001).

- Microscopic stool examination
- Examination of duodenal contents
- Small bowel biopsy
- Gastrointestinal radiology
- Immunodiagnosis
- Using DNA probe

2.13.1 Microscopic stool examination

The initial diagnostic approach for suspected giardiasis involves examining stool samples microscopically to identify cysts and trophozoites. *Giardia* cysts are frequently detected in the giardiasis stool samples, while trophozoites are seen very less. In giardiasis, symptomatic infection presence of trophozoites in the stool is often. (Goka et al. 1990). By light microscope, the stool samples can be examined either in their fresh state or after preservation with formalin and polyvinyl alcohol followed by staining with trichrome (Kim et al. 2005). The standardisation sensitivity examined using a single stool specimen is estimated to be approximately 50-70% (Goka et al. 1990). To enhance diagnostic accuracy, it is recommended to analyze two or three stool samples collected of different days considering that symptoms of giardiasis typically manifest 1 to 7 days prior to cyst excretion (Hiatt et al. 1995). Recent studies have shown that techniques such as immunomagnetic separation and sedimentation of cysts, followed by microscopy identification offer improved sensitivity and specificity compared to traditional methods (Massanet-Nicolau 2003).

2.13.2 Examination of intestinal fluid

In cases where multiple stool examinations yield negative results, the diagnosis can be established by examining the contents of the duodenum. Duodenal fluid can be collected using a duodenal tube or through the use of the Entero-Test, developed by the HEDECO Company based in Mountain View, California. With the Entero-Test, the patient ingests a gelatin capsule attached to a string, which facilitates the collection of duodenal fluid. Following a duration of several hours, the gelatin capsule is retrieved from the intestinal tract and subjected to

microscopic examination. The microscopic analysis of duodenal content has demonstrated higher sensitivity compared to the examination of stool samples (Hopper et al. 2003). Incorporating routine duodenal biopsies into the upper endoscopy procedure for pediatric patients has proven to be valuable in identifying additional pathological findings that may have otherwise gone undetected (Kori et al. 2003).

2.13.3 Small bowel biopsy

When conventional methods fail to provide a diagnosis of *Giardia* infection, small intestinal biopsies can offer improved diagnostic accuracy. These biopsies can be examined by sectioning and applying Giemsa staining. Typically, the *Giardia* parasite is commonly observed attaching to the border of microvillus, specifically within the crypts. While both small bowel biopsy and duodenal fluid tests exhibit high sensitivity and specificity, their clinical utility in diagnosis is limited due to their invasive nature (Bown et al. 1996).

2.13.4 Gastrointestinal radiology

Barium examination has been instrumental in detecting nonspecific intestinal changes that can aid in diagnosing giardiasis in approximately 20% of patients. These abnormalities serve as valuable indicators of the disease.

2.13.5 Immunodiagnosis

Compared to conventional staining methods, immunoassay procedures provide enhanced sensitivity and specificity in diagnosing giardiasis. These reagents are especially advantageous when screening a substantial number of patients, especially during an outbreak situation or when evaluating individuals with minimal symptoms. Immunoassay procedures offer a valuable tool for efficient and accurate screening in such scenarios. This is accomplished through two main approaches: i) detecting specific antibodies from patient's serum, and ii) detecting antigen in duodenal fluid and stool samples. Various immunoassay kits, employing techniques such as ELISA is commercially available to facilitate these diagnostic methods (Garcia et al. 2005). These kits provide convenient and reliable tools for identifying *Giardia* infections in clinical settings. Comparative studies (Aziz et al. 2001) comparing these immunoassay kits with microscopic and other direct methods, have demonstrated their higher specificity. Additionally sensitivity ranging from 94% to 99% for these kits, with a specificity

of 100% (Rashid et al. 2002). These findings indicate that the immunoassay kits offer excellent sensitivity and specificity, making them reliable tools for diagnosing *Giardia* infections.

2.13.6 Using DNA probe

In recent years, scientists have focused their efforts on developing highly sensitive and advanced diagnosis by nucleic acid detection. In 1994, Butcher et al. introduced a DNA probe specifically designed for diagnosing *Giardia*. Similarly, it has been reported total DNA probe development, capable of detecting *G. lamblia* (Allain et al. 2019). This probe has the ability to detect as little as 10 μg of *G. lamblia* DNA, as well as 104 trophozoites and 104 cysts. These advancements in nucleic acid detection have significantly enhanced the detection and diagnosis of *Giardia* infections. However, it should be noted that the total genomic DNA probe mentioned earlier exhibits cross-reactivity with a high amount (5μg) of *T. cruzi* DNA. To address this issue, researchers have developed various DNA probes specifically designed for detecting *Giardia* from stool samples (Dorsch et al. 2001). One successful approach has been the utilization of the non-transcribed giardial intergenic spacer region of the rRNA. This region has proven effective in differentiating *G. lamblia* from other enteric pathogens, providing a valuable tool for accurate and specific detection of *Giardia* infections.

The introduction of polymerase chain reaction (PCR) for the detection of these parasites and this technique specifically targeted the amplification of giardin gene (Shin et al. 2015). Since then, numerous PCR-based detection systems have emerged, offering improved sensitivity and specificity in diagnosing *Giardia* infections (Ghosh et al. 2000). PCR-based methods have revolutionized the field of *Giardia* detection, providing a powerful tool for accurate and efficient diagnosis. Real-time PCR, as reported by Verweij et al. in 2003, and colorimetric detection of PCR products (Lee et al. 2017) have also been utilized in *Giardia* detection. PCR-based methods offer advantages over other detection systems, particularly in terms of enhanced sensitivity and specificity. These techniques have significantly contributed to improving the accuracy and reliability of *Giardia* diagnosis.

2.13.7 Molecular Diagnosis -Nucleic acid Detection Methods

PCR-based techniques offer enhanced sensitivity compared to conventional and immunological assays when it comes to detecting *G. lamblia* in fecal samples. However, the sensitivity levels of published methods differ. Molecular methods are frequently confined to specialized laboratories; nevertheless, they are indispensable for identifying *G. lamblia* sub-assemblages

assemblages. The of assemblages A and B identification in human populations is crucial for understanding the epidemiology of the disease and determining potential transmission routes. It is worth noting that sequence of DNA-based surveys of *Giardia* have also detected assemblages A and B in nonhuman hosts, indicating the possibility of zoonotic transmission isolates of these assemblages (Caccio & Ryan 2008; Ortega-Pierres et al. 2009).

Molecular subtyping methods for *G. lamblia* are relatively less developed compered to other protozoan. However, specific genes have been utilized as targets for subtyping, including the glutamate dehydrogenase (gdh), β giardin (bg), elongation factor 1-alpha (ef-1), triose phosphate isomerase (tpi), small subunit ribosomal RNA (ssu-rRNA), and GLORF-C4 (C4) (Caccio & Ryan 2008). In their study, Caccio & Ryan 2008 emphasize two major challenges that can impede typing and subtyping of *G. lamblia*. Firstly, they note the presence heterogeneity of intra-isolate sequence, which refers to the existence of mixed templates that can influence the accurate each subtype assemblage. Secondly, they highlight will unreliable when different genetic markers are used, particularly when relying solely on a single genetic marker.

2.14 Treatment of Giardiasis

Over the past decade, considerable progress has been made in the treatment of giardiasis, as highlighted by Petri in 2003. Several effort have been undertaken to assess the susceptibility of this parasite to various drugs in laboratory settings. Currently, the drugs employed for the treatment of *giardia*sis include nitroimidazole metronidazole, Quinacrine, furazolidone and tinidazole (Minenoa et al. 2003).

2.14.1 Susceptibility of *G. lamblia* to aminoglycosides inhibitor

Aminoglycosides constitute a diverse and extensive category of antibiotics, with several members known to specifically target the 3' end of single-stranded (SS) RNA. The 3' end of SS rRNA plays a vital role in protein synthesis, including the binding of messenger RNA (mRNA) and transfer RNA (tRNA). Consequently, it has been identified as the specific site of action for multiple aminoglycoside antibiotics. Kasugamycin, the pioneering antibiotic associated with an rRNA target, exerts its effect on the two-methyl groups attached to A-1518 and A-1519 in bacterial cells. The rRNAs of *G. lamblia,* similar to those of other organisms, contain the AA dinucleotide, although the methylation status remains unknown. Studies have revealed that *Giardia* exhibits partial susceptibility to this drug (Edlind in 1989).

Hygromycin, an aminoglycoside, targets interaction between U-1495 and G-1494, these specific nucleotides are also found in *G. lamblia*, specifically within 1492-1506 highly conserved sequences. The WB strain of *G. lamblia* demonstrates high susceptibility to hygromycin, with an ID50 of 50 μg/ml. However, this drug lacks clinical utility due to its inability to exhibit selective toxicity (Edlind 1989). Paramomycin interacts at two distinct sites, which are spaced apart in terms of sequence but occupy the same position within the secondary structure. In Tetrahymena, susceptibility to this drug is conferred not by a specific sequence but rather by the 1409-1491 base pair. Interestingly, this particular base pair is also present in *G. lamblia*, and studies have demonstrated that the parasite exhibits high susceptibility to paramomycin (Edlind 1989).

Due to its high ID50, paramomycin is not commonly used for treatment. However, its limited absorption from the gut, similar to aminoglycosides, reduces the risk of systemic toxicity. As a result, paramomycin has been recommended for use during pregnancy. In bacteria, apramycin and kanamycin interact with A-1408 however, in *Giardia* this specific nucleotide is substituted by G, rendering these drugs ineffective against the parasite (Edlind 1989). Although the nucleotide G-1405, which interacts with gentamycin in bacterial SS rRNA, is also present in *Giardia*, the parasite shows significant resistance to this specific aminoglycoside (Edlind 1989). This suggests that additional factors or requirements are necessary for the activity of gentamicin against *G. lamblia*. Moreover, other aminoglycosides such as neomycin, sisomicin, ribostamycin, butirosin, and tobramycin have also been found to be ineffective against this parasite.

2.14.2. Susceptibility to Nitroheterocyclic Drugs

Currently, the control of *Giardia* infection is highly effective through the use of drugs belonging to the nitroimidazole group, with tinidazole and metronidazole being particularly noteworthy. These medications exhibit a wide range of activity against both protozoans and anaerobic bacteria, ensuring their effectiveness in managing *Giardia* infections. The treatment of giardiasis extensively relies on the use of metronidazole, which has proven to be highly effective, particularly when administered as a 5-day course (Adam 1991). Metronidazole exerts its mechanism of action by reducing the nitro group to generate metabolites that is nitro anion toxic radical through the involvement of reduced ferredoxin (Edwards 1993; Townson et al. 1994). This reduction process is facilitated by pyruvate ferredoxin oxidoreductase (PFOR) enzyme (Townson et al. 1996). According to Edwards in 1993, it has been suggested that the

toxic radicals generated by metronidazole could potentially bind to DNA, leading to the functional impairment of the entire cell. However, the proposed mechanism has not been definitively proven in nucleated organisms such as *Giardia*. Furthermore, Adam's observation in 1991 suggests that there are indications of potential toxic effects exerted by these radicals on the enzymes within the respiratory chain of the parasite. Notably, a decrease in pyruvate ferredoxin oxidoreductase (PFOR) activity has been linked to drug resistance in *Giardia*. The development of mechanisms in *G. lamblia* that enable it to tolerate oxidative stress can contribute to resistance against both oxygen and nitroheterocyclic drugs, which has implications for clinical control (Ansell et al. 2015). It should be noted that the drug itself exhibits mutagenic properties in bacteria, and high doses administered for prolonged periods have been found to be carcinogenic in mice. In humans, common side effects during therapy include nausea and general malaise.

Single dose of tinidazole is highly effective and is exceptionally well tolerated, making it the preferred drug of choice. However, tinidazole induces genetic damage in human lymphocytes (Lopez Nigro et al. 2001). Furazolidone, which belongs to the nitroheterocyclic drug family, undergoes reduction to cytotoxic compounds in a manner similar to the 5'-nitroimidazoles. However, this reduction process does not occur via ferredoxin (Brown et al. 1996). In *Giardia*, the activation of furazolidone is facilitated by the enzyme NADH oxidase (Brown et al. 1996) and this enzyme does not seem to play the role resistance of furazolidone. Instead, it appears that resistance to furazolidone in *Giardia* involves alterations in the membrane structure.

2.14.3. Susceptibility to Benzimidazoles

Mebendazole, which belongs to the benzimidazole class of drugs, acts as a broad-spectrum antihelminthic agent and exhibits efficacy against *Giardia* by interacting with β-tubulin. Albendazole, another benzimidazole compound, has been found to possess *G. lamblia* in vitro (Meloni et al. 1990). The study compared two different methods for assessing the *G. lamblia* trophozoites susceptibility to albendazole and metronidazole. The first method, known as Meloni's method, measured the parasites adherence loss to surfaces, while the second method, the Hill method, assessed the reduction in parasite division capacity. The results suggested that the adherence method exhibited greater sensitivity than the multiplication method for detecting the inhibitory effects of albendazole at low and moderate concentrations. On the other hand, in multiplication method the metronidazole is more sensitive when it comes to drug high

inhibitory concentrations. However, for screening the IC50 (half maximal inhibitory concentration), both methods have shown effectiveness. It is worth noting that the adherence inhibition method demonstrates even better performance specifically for benzimidazole-like drugs (Cruz et al. in 2003). In cases of albendazole and metronidazole-resistant giardiasis, successful treatment has been achieved using nitazoxanide, particularly in patients with acquired immunodeficiency syndrome (Abboud et al. 2001). According to a study by Martinez et al. in 2015, analogues of Albendazole (Abz) and Mebendazole (Mbz) exhibit comparable activity to Metronidazole as antiprotozoal agents against *G. lamblia*. Additionally, in a separate study conducted by Navarrete-Vazquez et al. in 2003, it was found that these Albendazole and Mebendazole analogues possess similar efficacy to Metronidazole against *G. lamblia*. Additional investigations reveal that Albendazole causes DNA damage and induces oxidative stress in *Giardia* (Martinez et al. 2015).

Secnidazole, which is another derivative of Benzimidazole, can be used as a treatment for giardiasis and has shown effectiveness in eliminating chronic *Giardia* infections (Escobedo et al. 2003). In separate studies conducted by O'Handley et al. in 2001 and Keith et al. in 2003, Fenbendazole has demonstrated remarkable efficacy in treating giardiasis by effectively eliminating *Giardia* trophozoites specifically within the small intestine (O'Handley et al. 2001; Keith et al. 2003). Quinacrine, known as a flavoantagonist, is believed to suppress the oxidation of NADPH in *G. lamblia*. It has been found to be highly effective against giardiasis; however, it is associated with common gastrointestinal side effects (Upcroft et al. 1996).

2.14.4. Other agents

Several other agents have been shown to exhibit in vitro activity against *Giardia*. These include chloroquine, rifampin, pyrimethamine, azithromycin, mefloquine, and doxycycline. Additionally, ciprofloxacin has been found to have cytotoxic effects against *Giardia* serve as an alternative treatment option for giardiasis, especially in cases where the infection shows resistance to other antibiotics (Sousa et al. 2001). When patients do not initially respond to treatment for giardiasis, they often exhibit a positive response when given a second course of treatment using either the original agent or an alternative one. A notable approach for effective treatment involves combining quinacrine and metronidazole, which has shown significant efficacy in combating giardiasis.

2.15. Cultivation of Parasite

In 1970, Meyer *G. lamblia* trophozoites successfully cultured obtained from a rabbit, chinchilla, and cat in an axenic environment, which means they were grown without the presence of exogenous cells. A modified medium known as HSP-1 was introduced in 1976 by Meyer, which consisted of glucose, phytone peptone, Hanks solution, L-cysteine HCl and human serum. This modification aimed to improve the cultivation of *G. lamblia* trophozoites. In the study, the first human isolate of *G. lamblia*, named Portland-1 (P-1), was reported. P-1, along with the WB isolate, which was obtained from human symptomatic likely infected in Afghanistan (Smith et al. 1982) that belong from the same genotype. These isolates have been extensively utilized in various studies related to *G. lamblia*. Over time, the growth medium has undergone modifications, and currently, the most widely used modified TYI-S-33 medium (Keister 1983). Axenic growth of *G. lamblia* requires specific conditions, including a low concentration of oxygen, high cysteine concentration, and lipids from serum. During cultured at 37°C, the adherence of trophozoites to the glass surface of the growth container. The adherence of *G. lamblia* trophozoites to the glass surface is dependent on the metabolic process of glycolysis and the contraction of proteins within the ventral disk (Feely et al. in 1982). Other species of *Giardia*, such as *G. agilis* and *G. muris*, have not been successfully grown in axenic culture to date.

CHAPTER-III

MATERIALS

3. Materials

3.1. Different growth mediums

3.1.1. Parasite growth medium

3.1.1.1. Modified TYIS-33 medium

Component	Amount (gm)
K_2HPO_2	1.0
KH_2PO_4	0.6
L-cysteine-HCl (monohydrate)	1.0
Ferric ammonium citrate	0.022
Sodium chloride	2.0
D-glucose	10.0
Ascorbic acid	0.2
Casitone	20.0
Yeast extract	10.0
Double distilled water upto	870 ml

The pH of the medium was adjusted to 6.8 with 1N NaOH. Subsequently, the medium underwent filtration using Whatman No. 1 filter paper to eliminate undissolved particulate matter and was sterilized by passing through a pre-autoclaved 0.22 µm membrane filter system (Millipore, USA).

3.1.1.2. Vitamin-Tween mixture

3.1.1.2.1. Solution B

The vitamin mixture NCTC was prepared in accordance with the original description provided by Evans et al. in 1956 for its application in tissue culture medium.

3.1.1.2.2. Solution B

Forty milligrams of vitamin B12 (Sigma) were dissolved in double-distilled water, and the final volume was adjusted to 100 ml.

3.1.1.2.3. Solution C

A hundred milligrams of D-L-6, 8-thioctic acid (oxidized form) (Sigma) were dissolved in a small volume of ethanol, and the final volume was adjusted to 100 ml with double-distilled water.

3.1.1.2.4. Solution D

Fifty grams (w/v) of Tween-80 were dissolved in ethanol, and the volume was adjusted to 100 ml with double-distilled water.

3.1.1.2.5. Complete Vitamin-Tween mixture working dilution

To 1 litre of stock solution, 12 ml of solution B, 4 ml each of solutions C and D and 180 ml of double distilled water were added. The solution was then sterilized by passing through 0.22 µM membrane filters (Millipore, USA) and stored at -20°C.

3.1.1.3. Bovine serum

Bovine blood freshly collected from the slaughterhouse was allowed to clot by incubating it at 37°C for 1 hour, followed by overnight incubation at 4°C. The unclotted material underwent centrifugation at 10,000g for 10 minutes to pellet down residual red blood corpuscles (RBC), and the resulting clear serum was collected in a fresh container.The serum was subjected to decomplementation at 56°C for 30 minutes, then sterilized by filtration through 0.22 µm sterile membranes (Millipore) and stored in aliquots at -20°C until needed.

3.1.1.4. Antibiotic solution

Penicillin (Benzylpenicillin Injection IP, Alembic Limited: 10,000,000 IU) and streptomycin (Ambistyrin S®, Sarabhai Piramal: 1 gm) were dissolved in 10 ml of sterile triple-distilled water.

Complete Growth Medium

The components mentioned above were mixed at specific concentrations to formulate the complete growth medium for parasite culture. The composition of 100 ml of the complete TYIS-33 medium is as follows –

Components	Volume (ml)
Autoclaved TYIS-33 growth medium	87
Sterilized heat inactivated bovine serum	10 ml
Complete vitamin mixture	3ml
Antibiotic solution	120 µl

The complete growth medium was aseptically dispensed in 9 ml volumes into sterile screw-capped bottles (15X125 mm, Borosil, India). The medium was then incubated at 37°C overnight to confirm its sterility.

3.1.2. Luria broth (LB) and agar (LA)

Twenty grams of Luria Broth mixture from DIFCO were measured and added to triple-distilled water and the volume was adjusted to 1 liter. Luria agar was prepared by adding fifteen grams of agar per liter of LB.

3.1.2.1. SOC medium

The composition of SOC medium is as follows:

- 2% tryptone
- 0.5% yeast extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl2
- 10 mM MgSO4
- 20 mM glucose

3.2. Solutions and buffers

3.2.1 Solutions for genomic DNA isolation

3.2.1.1. Lysis buffer

The concentrations of the components in the utilized lysis buffer are as follows:

- 20 mM Tris-HCl (pH 8)
- 50 mM EDTA solution
- 0.5% Sarcosyl
- 500 µg/ml Proteinase K.

3.2.1.2. CTAB/NaCl solution

4.1 gm of NaCl were dissolved in 80 ml of double-distilled water. Ten milligrams of CTAB (Hexadecyltrimethylammonium bromide) were added and dissolved with continuous stirring and heating at 65°C.

3.2.1.3. Phenol: Chloroform: Isoamyl alcohol

The solution consists of a mixture containing 50 mM Tris-HCl (pH 8), saturated phenol, chloroform, and isoamyl alcohol in a ratio of 25:24:1, respectively**.**

3.2.1.4. 3M Sodium acetate

24.6 grams of sodium acetate were dissolved in the minimum volume of triple-distilled water, and the pH was adjusted by adding glacial acetic acid. Finally, the volume was adjusted.

3.2.1.5. RNase A

A 10 mg/ml solution of RNase A was prepared following the manufacturer's protocol.

3.2.2. Solutions for plasmid DNA isolation

3.2.2.1. Solution I (Glucose-Tris-EDTA)

The concentration of the components in this solution was as follows:

- 50 mM Glucose
- 25 mM Tris-HCl (pH 8)

- 1 mM EDTA

3.2.2.2. Solution II (SDS-NaOH)

The composition of this solution was:

- 0.2 M NaOH
- 1% SDS

3.2.2.3. Solution III (Sodium acetate)

24.6 gm of sodium acetate were dissolved in the minimum volume of triple-distilled water, and the pH was adjusted by adding glacial acetic acid. Finally, the volume was adjusted.

3.2.2.3. TE Buffer

- 10 mM Tris-HCl
- 1 mM EDTA (pH 8.0)

3.2.2.3. RNase A and Phenol: Chloroform: Isoamyl alcohol solution

Comparable to the solution detailed in sections 3.2.1.5 and 3.2.1.3.

3.2.3. Solutions and buffers for gel electrophoresis

3.2.3.1. TBE for agarose gel electrophoresis of DNA

The concentration of the components in 10X TBE was as follows –

- 89mM Tris,
- 89mM borate,
- 2mM EDTA (pH 8)

3.2.3.2. Agarose solution

The required quantity of agarose powder was suspended in 0.5X TBE, melted, cooled to 60°C, and then poured into the casting block.

3.2.3.2. Ethidium bromide (EtBr) solution

To prepare this solution, 0.1 gm of EtBr was dissolved in 10 ml of triple-distilled water.

3.2.3.2. Buffers for polyacrylamide gel electrophoresis

3.2.3.2.1. 10X PBS (Phosphate-buffered saline, 100 mM)

- 0.58 M Na2HPO4
- 0.17 M NaH2PO4
- 0.69 M NaCl

3.2.3.2.2. Cell lysis buffer

The lysis buffer was prepared by dissolving the following components in triple-distilled water:

- 100 mM NaCl
- 10 mM Tris-HCl (pH 8)
- 10 mM EDTA (pH 8)

3.2.3.2.3. 100mg/ml proteinase K

- 100 mg proteinase K
- 1 ml dH_20

3.2.3.2.4. Shearing buffer

- 10 mM Tris-Cl,
- 1 mM EDTA, pH 8.0
- 20% glycerol

3.2.3.2.5. Shearing buffer 6X gel-loading buffer

- 0.25% bromophenol blue
- 40% (w/v) sucrose in water

3.2.3.2.6. 1X Protein sample buffer

- 10 mM Tris-HCl, pH 7.9
- 10 mM MgCl2,
- 50 mM NaCl,
- 1 mM dithiothreitol (DTT)

3.2.3.2.7. 10X SDS running buffer

- 25 mM Tris base
- 250 mM Glycine
- 0.1% SDS

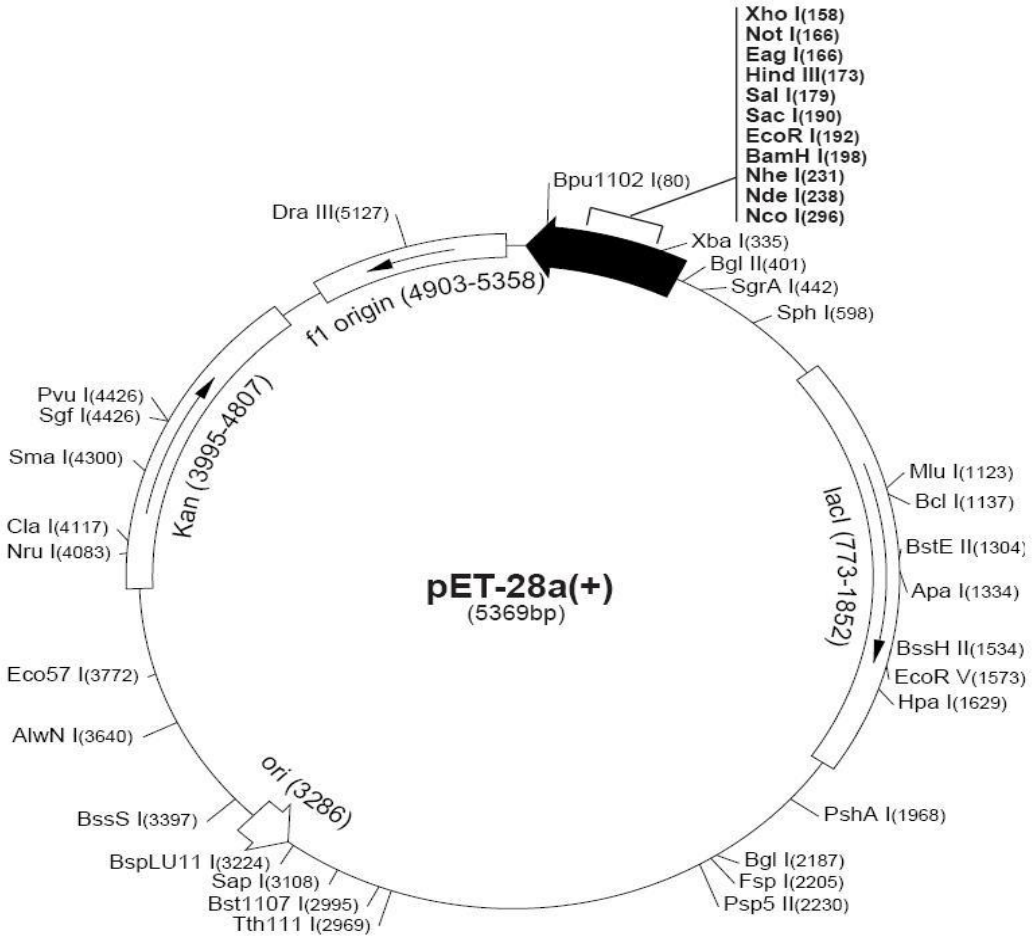
3.2.3.2.8. Transfer buffer

- 25mM Tris-HCl
- 192 mM Glycine
- 20% (V/V) ethanol

3.2.3.2.9. Tris buffer saline–Tween 20 (TBS-T)

- 10mM Tris-HCl
- 150 mM NaCl, pH 7.4
- 0.05% (v/v) Tween 20

3.3. pET 28a vector for Cloning and Expression



CHAPTER-IV

STUDIES THE CYTOTOXIC ACTIVITY OF PLANT EXTRACT AGAINST *Giardia* IN VITRO

Studies the cytotoxic activity of plant extract against *Giardia* in vitro

4.1. Background

Giardiasis is a common intestinal protozoal infection that is widely prevalent in humans (Gardner et al. 2001). It is caused by *Giardia lamblia*, a flagellated protozoan that primarily affects the small intestine of both humans and animals (Adam 2001). An estimated 280- million giardiasis cases have been diagnosed worldwide every year, and the prevalence rates of the infection may vary between 20–30% in developing countries and 2–5% in developed countries, respectively (Ankarklev et al. 2010). The clinical presentation of giardiasis in humans can vary, ranging from asymptomatic cases to those experiencing symptoms such as vomiting, nausea, diarrhoea, and abdominal cramps during the acute phase. In chronic cases, giardiasis has been associated with various health complications, including IgA deficiencies, malnutrition, and immunosuppression (Escobedo et al. 2018). Additionally, giardiasis has been linked to growth retraction, arthritis, cognitive impairment, pulmonary infiltrate irritable bowel syndrome and urticaria (Dyab et al. 2016). In children, severe infections can lead to malnutrition and affect both mental and physical development (Hotez et al. 2004). In individuals with HIV/AIDS, giardiasis can manifest as either acute or chronic diarrhea (Merchant et al. 1996). The primary treatment option for giardiasis is Metronidazole, which is commonly used as the first-line therapy. Other drugs such as Tinidazole, Albendazole, and Furazolidone are also utilized (Alizadeh et al. 2006; Brandelli et al. 2009; Moerch et al. 2008; Tracy et al. 1996). However, it is important to note that treatment of giardiasis can be challenging due to the frequent occurrence of undesirable side effects, treatment failures, and the emergence of drug resistance (Savioli et al. 2006; Alizadeh et al. 2006; Tracy et al. 1996). Metronidazole, commonly used in the treatment of giardiasis, can lead to various side effects such as vomiting, nausea, lethargy, diarrhoea, loss of appetite, anemia, weakness, blood in the urine, head tilt, seizures, disorientation, and stumbling. Additionally, it has the potential to cause liver disease, resulting in yellowing of the skin, gums and eyes. Nerve damage is also a possible adverse effect, and studies have confirmed the mutagenic effects of this drug in certain bacteria and animal models (Tracy et al. 1996; Johnson et al. 1993; Lemee et al. 2000; Voolmann et al. 1993) Furthermore, metronidazole has been associated with reported carcinogenic, teratogenic, and embryogenic properties (Upcroft et al. 1999). Given these

concerns, it is imperative to explore and develop new therapeutic alternatives. Exploring traditional indigenous medicine and leveraging ethnobotanical knowledge represents a promising approach to the discovery of novel therapeutic products.

Andrographis paniculata (*Ap*) is one of the most important traditional medicinal plant that is used widely throughout the world. Many countries like India, Bangladesh, China, Pakistan, Hong Kong, Philippines, Thailand, Malaysia and Indonesia used this plant as traditional medicine (Akbar et al. 2011; Kabir et al. 2014; Hossain *et al.*, 2014; Jayakumar *et al.*, 2013). This plant possesses numerous therapeutic properties, including the treatment of conditions such as diabetes, dysentery, fever, malaria, insect bites, and snakebites (Hossain et al. 2014). While the entire plant is utilized as a therapeutic agent, the aerial parts of the plant, specifically the leaves and stem, are the most commonly used portions. This plant contains various chemical compounds, including diterpenoids, glycosides, diterpenes, flavonoids, lactones, and flavonoid glycosides. This plant possesses a wide range of pharmacological activities such as antihepatitic (Jayakumar et al. 2013), antidiarrheal (Gupta et al. 1990), anti-inflammatory, anti-allergic, immunostimulatory, antidiabetic, antioxidant (Okhuarobo et al. 2014), antimalarial (Misra et al. 1992), hepatoprotective, anticancer, cardiovascular, antihyperglycemic (Hossain et al. 2014), and anti-HIV (Nanduri et al. 2004) activities.

The purpose of this study is to investigate the protective effect of the ethanolic extract of *A. paniculata* on the protozoan parasite *Giardia*.

3.2. Methodology

3.2.1. Cell Culture

G. lamblia Portland1 strain (ATCCR 30888) was maintained in TYIS-33 medium supplemented with penicillin streptomycin and 10% adult bovine serum (Diamond et al. 1978). The medium pH was adjusted to 6.8 during the filter sterilization using 0.22µm. The culture was grown axenically under the anaerobic condition at 37°C. After 24 h of incubation, cell growth and viability were examined.

4.2.2. Preparation of extract

The *A. paniculata* ethanolic extract was prepared by following the method described by Abd-Elhamid et al. 2021. In this process, 200 grams of powdered plant material were mixed with 1 litre of 95% ethanol and after maceration, the mixture was filtered. The combined filtrates were then evaporated until dryness, resulting in the crude dry ethanoic extract. The obtained dry

extract was stored at 4 °C for future use (Abd-Elhamid et al. 2021). To prepare aqueous extract 500 g powder was mixed with 1000 ml of boiled distilled water and for 120 minutes with continuous shaking. The containers were left overnight, and the mixture was subsequently filtered through Whatman filter paper. The resulting filtrate underwent evaporation and dry residue was stored at 4 °C (NM et al. 2019). For the chloroform extraction, 70 g of the dry powder was mixed with 350 ml of pure chloroform using a magnetic stirrer for 60 minutes. The solution was left at room temperature for 24 hours, stirred again, and filtered. The solvent was evaporated and the semisolid material was stored at 4 °C for future use (Golami et al. 2016).

4.2.3. Seeding of *Giardia lamblia* trophozoites

The process for collecting trophozoites involved several steps. First, the parasites were subjected to a 10-minute cold shock to loosen the cells from a glass tube. This was followed by a 10-minute centrifugation at 2000 rpm to separate the cells from any residual fluid. Next, the cells were washed three times with PBS at a pH of 7.2 and a temperature of 4°C. The cell population was then counted using a hemocytometer slide to determine the number of cells present (Carvalho et al. 2014). Finally, 2 X 10^6 cells were added to 12 ml of modified TYIS-33 medium and incubated for 24 hours at 37°C. The goal was to reach 80-90% confluency with the cells in the log phase of growth. Once this was achieved, the old medium containing detached and dead cells was discarded, and the cultures were replenished with fresh growth medium. Treatment was then administered.

4.2.4. Antiprotozoal assay

To evaluate the effectiveness of *Ap* extracts against *Giardia* trophozoites, various types of extracts-such as aqueous, ethanolic and chloroformic were introduced to trophozoites during their logarithmic growth phase. Various concentrations and durations of the extract were treated in culture tubes that were nearly 90% confluent. The concentrations employed for treatment were 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml and 125µg/ml, with a duration of 24 hours. The IC_{50} concentrations of various extracts were determined, and subsequently, *Giardia* cells were exposed to these concentrations for varying durations: 8 hours, 12 hours, 24 hours, 48 hours and 72 hours. This experimental setup was performed in triplicate for each condition. Various concentrations of aqueous solution were used to prepare the aqueous extract, while ethanolic and chloroformic extracts were prepared using DMSO as the solvent. Consequently, DMSO was employed as the control for treating the culture medium with ethanolic and

chloroformic extracts across all instances. In contrast, when working with the aqueous extract, an equal volume of water was introduced into the culture tube to function as the negative control.

Following the completion of each treatment, the parasites were separated by cooling, and subsequently, the flow cytometry assay was employed to determine the percentage of cell death. In trypan blue assay, the parasites were washed with PBS and subsequently incubated in a 0.4% trypan blue solution at room temperature for 5 minutes. Finally, the parasites were counted under a microscope using a hemocytometer (Carvalho et al. 2014). The flow cytrometry assay was performed by using Annexin V-FITC Kit (BD Bioscience) following manufacturing protocols. The treated and control cells were thoroughly rinsed with chilled PBS and centrifuged at 2000 rpm for 8 minutes on two occasions. After the second washing, the cell pellet was resuspended in 1ml of chilled 1X binding buffer at a concentration of $2x10^6$ cells. 5µl of Annexin V and 5µl of propidium iodide (50µg/ml) solution were added and incubated for 1 hour in the dark (Martínez-Espinosa et al. 2015). Finally, the cells were resuspended with 400µl 1X binding buffer and incubated at room temperature for 15 min. The samples were protected from light and analysed using flow cytometry (BD FACSAria^{TM} II). The IC_{50} value for various extracts of *A. paniculata* on *Giardia* was calculated using the GraphPad prism v.8.4.2, CA, USA.

4.2.5. Adherence Property

Giardia trophozoites (4 x 10^4/ml) were cultivated on a glass tube under microaerophilic conditions at 37°C. The culture was prepared using TYIS-33 medium with the addition of 10% adult bovine serum. The plant extracts were added to the culture at varying concentrations ranging from 25µg/ml to 125µg/ml, with one well serving as the control without the extract. The culture was incubated for 6 hours, after the following incubation, the culture medium containing unattached cells was removed and counted using a haemocytometer (Sousa et al. 2001). This study assessed all three extracts derived from *Ap*.

4.2.6. Scanning Electron Microscopy (SEM)

To conduct the experiment, cells were treated with various *A. paniculata* extracts (such as aqueous, chlorformic and ethanolic extracts) at the IC_{50} concentration for 24 hours. After the treatment, the cells were collected, washed with PBS buffer (pH 7.2) and fixed in cacodylate buffer at 4°C overnight. The fixed samples were dehydrated using a series of increasing concentrations of alcohol (30%, 50%, 70%, 90%, and 100%) and then treated with 2 ml of

hexamethyl disilaxane to remove any residual alcohol from the samples without causing structural damage (Frontera et al. 2018). This was followed by an additional hour of incubation after adding 1 ml of hexamethyl disilazane. The samples were kept in a fume hood overnight. For SEM imaging, the sample was mounted on a stub using conductive adhesive and coated with a thin layer of gold using evaporation techniques. This process enhances the conductivity of the samples, which is essential for SEM imaging.

4.2.7. Cell cycle analysis

The *Giardia* trophozoite cells were collected and washed with PBS (pH-7.2) by centrifuging at 1500rpm for 10 minutes at 4℃. Then, the cells were dissolved in 70% ethanol and fixed overnight at 4℃ (Martínez-Espinosa et al., 2015). To avoid cell clumping during fixation, 100µl of 70% ethanol was repeatedly added while vortexing. The Cycle FXCycle^{TM}PI/RNase staining solution kit (ThermoFisher) was used to determine the cell-cycle phase distributions. After centrifugation at 1500 rpm for 10 minutes, the cells were treated with RNase solution and incubated for an hour at 37℃ following the manufacturer's protocol. Finally, the cell cycle analysis was performed using a flow cytometer (BD FACSAria^{TM} II).

4.2.8. DNA Degradation

The *Giardia* trophozoites were exposed to IC_{50} concentrations of plant extract and culture was centrifuged at a low speed 1500rpm for 5 minutes. The pellets were subsequently washed by resuspending in PBS (pH 7.2). To fix the cells, a 4% formaldehyde solution was used, and then the cells were incubated with 0.1% Triton X-100 (Ordoñez-Quiroz et al. 2018). For staining the nuclei, a DAPI (4',6-diamidino-2-phenylindole) solution was added directly to the resuspended pellet, achieving a final concentration of 1-10 µg/ml. The pellet was gently mixed to ensure the even distribution of DAPI throughout the sample. After adding the DAPI stock solution to the resuspended pellet, the sample was incubated in a dark at room temperature for 30 minutes to permit the DAPI dye to penetrate the nuclei of the parasites. After the incubation period, the sample was centrifuged and the excess DAPI was removed by washing the pellet with PBS. This washing step was repeated at least three times, with each cycle involving centrifugation at a low speed for 5 minutes. Subsequently, the washed pellet was used to prepare coverslip-mounted samples on glass slides. Finally, the prepared slides were subjected to imaging using a Confocal Microscope (Zeiss LSM 710) to visualize the stained parasites.

4.2.9. ROS generation

To assess the production of reactive oxygen species (ROS) in trophozoites cells, we utilized a cell-permeable fluorescent dye called H2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate). This dye undergoes hydrolysis by nonspecific intracellular esterases and gets oxidized by cellular peroxides, resulting in the formation of a fluorescent compound known as DCF (2', 7'-dichlorofluorescein). Therefore, the intensity of fluorescence directly correlates with the amount of ROS generated by the cells. In this experiment, trophozoite cells were exposed to the IC_{50} concentration of the extracts for 24 hours. Afterwards, the cells were rinsed with PBS and then incubated with H2DCFDA (5μM) in PBS for 30 minutes at 37°C in the absence of light (Raj et al., 2014). Subsequently, the cells were detached, washed, and suspended in PBS for analysis of DCF fluorescence. We performed data acquisition using a flow cytometer (BD FACSAria^{TM} II), with an emission wavelength of 517 nm and an excitation wavelength of 492 nm.

4.3. Results

We conducted an assessment of the antigiardial properties of three different extracts obtained from *A. paniculata*. Specifically, we examined the aqueous, ethanolic, and chloroformic extracts. Notably, our findings revealed that the ethanolic extract exhibited substantial antigiardial effects. However, the aqueous and chloroformic extracts did not exhibit noteworthy antigiardial activity; consequently, we omitted the data associated with these extracts. In the following results section, we present the outcomes of utilizing the ethanolic extract as an agent against *Giardia*, highlighting its effects.

4.3.1. Efficacy and mode of action of ethanolic extract against *Giardia lamblia*

The percentage of cell death increased with an increasing concentration of ethanolic extract. The data presented in Fig. 4.1 displays the mean values obtained from triplicate measurements using trypan blue and flow cytometry assay. The cells were labelled with FITC-PI and subjected to various concentrations of treatment, ranging from 25μg/ml to 125μg/ml. The average percentage of cell death was 25.86% when *A. paniculata* extract was present at a concentration of 25μg/ml. When the concentration was increased to 50μg/ml, the average percentage of cell death increased to 44.04%. Similarly, increasing the concentration to 75μg/ml resulted in an average cell death rate of 59.08%, at a concentration of 100μg/ml, the

average cell death rate was 71.66% and the concentration at 125µg/ml showed 90.21%. (Fig. 4.1). Therefore, the ethanolic extract of this plant showed potent activity against *Giardia* trophozoites. The IC_{50} growth inhibition of *A. paniculata* extracts against *Giardia* cells after a 24-hours incubation was determined to be 51.26µg/ml (95% CI 65.35).

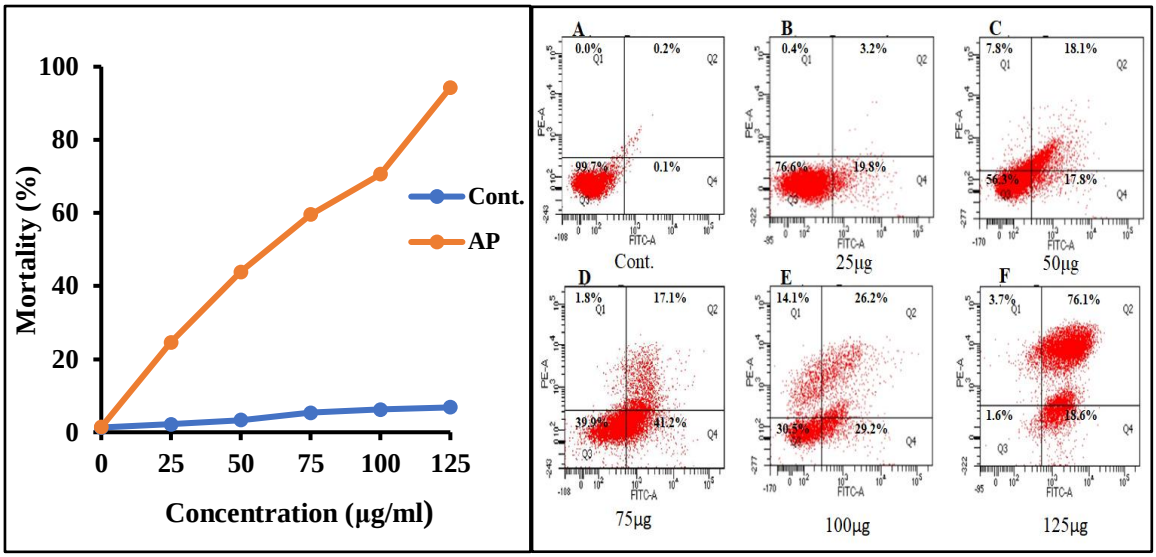


Figure 4.1: The figure depicts the results of a flow cytometry and trypan blue assay that were treated with different concentrations of *Ap* extract. The panel includes a negative control (1A) and five treatment groups, consisting of *Giardia* cells treated with various concentrations of Ap extract (25µg/ml in 1B, 50µg/ml in 1C, 75µg/ml in 1D, 100µg/ml in 1E, and 125µg/ml in 1F). The cells were categorized based on their response to treatment, with quadrant Q1 representing necrotic cells, quadrant Q2 representing late apoptotic cells, quadrant Q3 representing living cells, and quadrant Q4 representing early apoptotic cells.

The mortality rate of *G. lamblia* trophozoites in the presence of the plant ethanolic extract at IC_{50} concentration was observed at different time intervals. Initially, after 2 hours of treatment, there was no significant impact on the trophozoites. However, after 8-12 hours of treatment, the plant extract displayed higher activity and resulted in increased trophozoite death frequency with time. The cell death activity was found to increase gradually, with 19.3% cell death observed at 8 hours and 50.2% at 24 hours of treatment (Fig. 4. 2). Moreover, at the 72-hour time point, the maximum level of activity was observed, with 99.2% of cells undergoing death after exposure to the IC_{50} concentration.

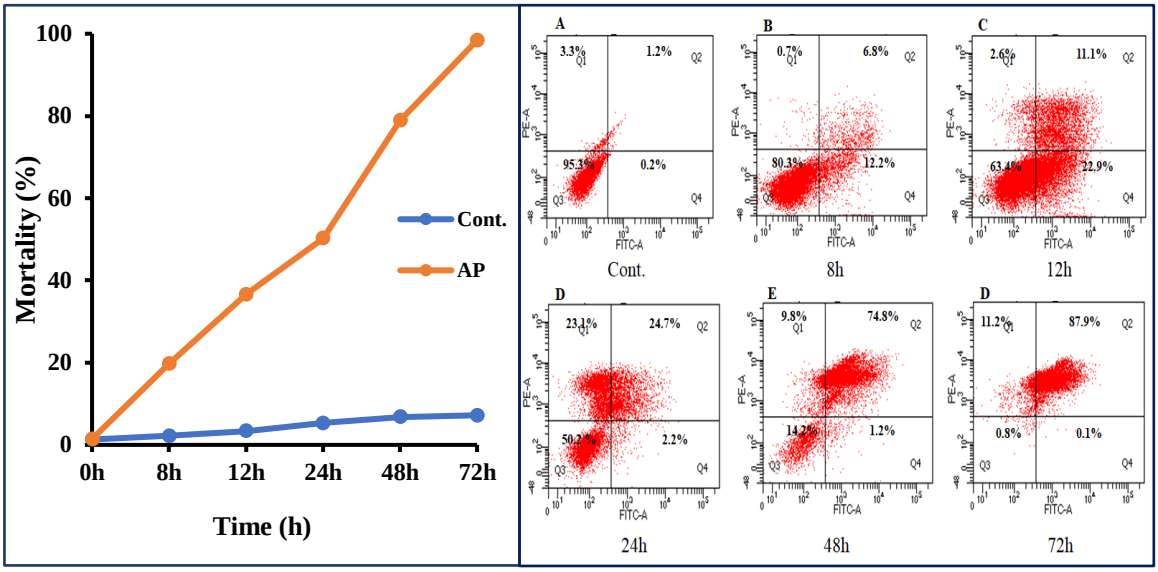


Figure 4.2: The figure depicts the mortality rate of *G. lamblia* trophozoites that were exposed to IC_{50} concentration for different times ranging from 0-72 hours of incubation. The panel includes a negative control (1A) and five treatment groups, consisting of *Giardia* cells treated with various time of *Ap* extract 8h in B, 12h in C, 24 in D, 48 in E, and 72 in F). The cells were categorized based on their response to treatment, with quadrant Q1 representing necrotic cells, quadrant Q2 representing late apoptotic cells, quadrant Q3 representing living cells, and quadrant Q4 representing early apoptotic cells.

4.3.2. Attachment of *Giardia lamblia*

The results of the study indicate that the ethanolic extract has a significant effect on the attachment of *Giardia* trophozoites to enterocytes, which is an essential step for the colonization of the small intestine. The attachment of the trophozoites to the tube wall was reduced after treatment with the plant extract, and this reduction was proportional to the concentration of the extract. After an incubation period of 6 hours, the percentage of unattached cells obtained were 16.38%, 34.80%, 44.78% and 68.18% at extract concentrations of 25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml respectively. At a concentration of 125µg/ml, the study found that 82.95% of the trophozoites did not adhere to the glass after 6 hours of treatment, suggesting that the plant extract has the potential to be an effective agent against *Giardia* infection (Fig. 4.3).

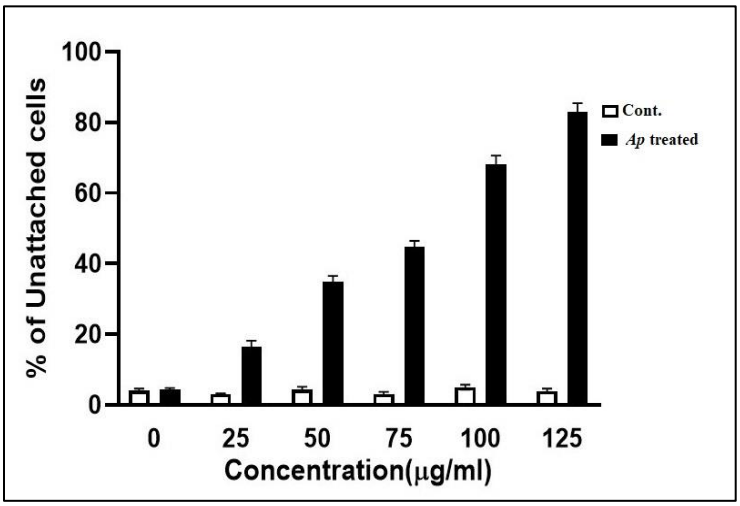


Figure 4.3: The graph illustrates the mean reduction in adherence of *Giardia* trophozoites after exposure to *A. paniculata* extracts at varying concentrations ranging from 25μg/ml to 125μg/ml. The y-axis represents the percentage of inhibition of adherence, while the x-axis shows the concentration of *Ap* extracts in mg/ml. The graph shows the average of triplicate experiments

4.3.3. DNA Degradation

DNA degradation study demonstrated that treatment with the extract at the IC_{50} concentration resulted in noticeable DNA damage, evident from a positive signal indicating nucleus damage in trophozoites. Figure 4.4 clearly shows the noticeable difference between the control cells and the cells treated with *Ap*. Control cells appear normal with intact nuclei and no DNA damage, as seen in DAPI staining. However, Ap-treated cells show evident DNA damage. The staining was observed throughout the entire body of the trophozoites. These results further support the notion that the plant extract possesses cytotoxic effects on trophozoites.

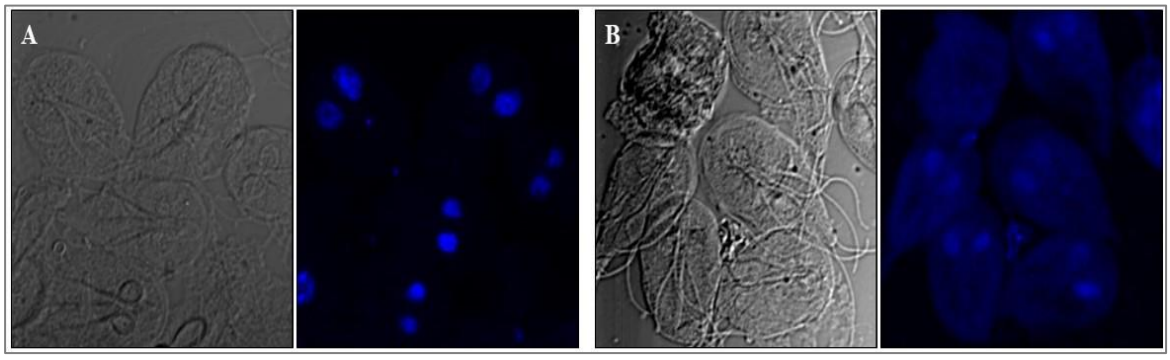


Figure 4.4: The figure shows the results of a DNA degradation analysis (A-B). In control living cells where no DNA degradation was observed (A). However, there was significant DNA damage observed after being treated with *Ap* extract (B).

4.3.4. Cell cycle Arrest

The nuclei of the trophozoites were stained with PI to determine if the plant extract inhibit the cell cycle progression of the treated trophozoites. The results, shown in Fig. 4. 5, revealed that the IC_{50} concentration of the extract after different time interval resulted in a significant decrease in the G2/M subpopulation (40.4% in control cells and 29.1%, 19.2 and 4.6% extract-treated cells) and increase the subG0 subpopulation (2.6% in control cells and 3.2%, 39.4% and 81.2% in extract treated cells). Following a 48-hour period, the G2/M subpopulation decreased of 4.6%, while the sub-G0 subpopulation exhibited an increase of 81.2% among the cells that were subjected to extract treatment. These findings indicate that the plant extract has a cytotoxic effect at IC_{50} concentration, leading to the sub-G0 arrest and early apoptosis.

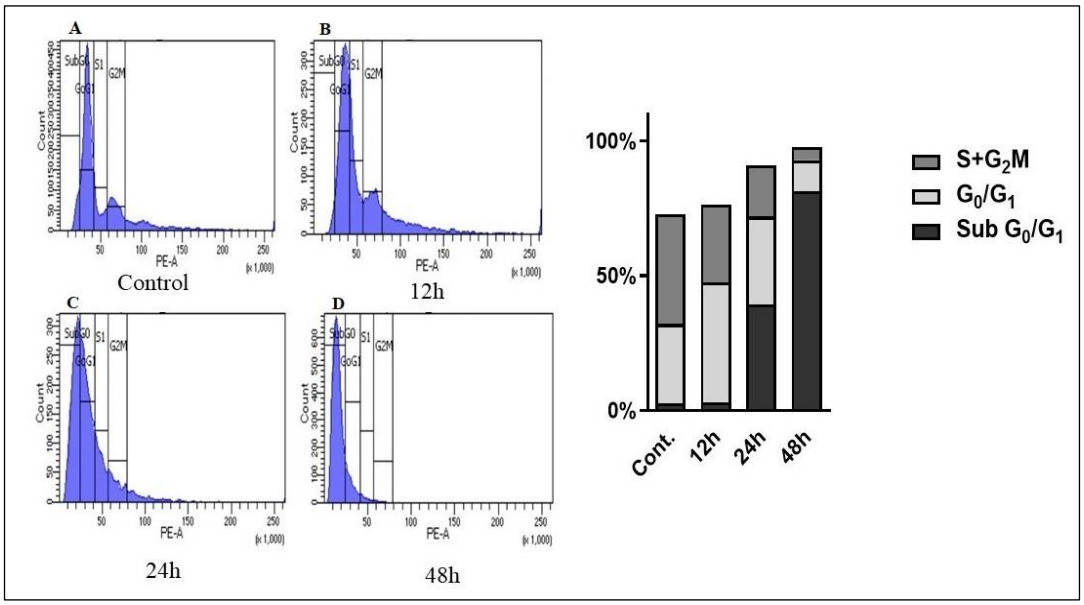


Figure 4.5: The effect of *Ap* extract treatment on the cell cycle of *Giardia* trophozoites using flow cytometry analysis: Control cells showing the trophozoites contained 4N DNA content in the G1 phase (A). Cells were exposed to the IC_{50} concentration of the extract and incubated for different time points: 12h (B), 24h (C), and 48h (D). The extract treatment caused cell cycle arrest, leading to a significant arrested the cells in subG0 phase.

4.3.5. Cell Morphology study

We observed that the shape and size of *Giardia* cells could be altered after the ethanolic extract treatment. This can occur through various mechanisms such as interference with the cell membrane, changes in gene expression or signalling pathways, and induction of cellular stress. The plant extract may also cause changes in *Giardia* morphology through multiple mechanisms, including effects on motility, oxidative stress, and alteration of gene expression.

Fig. 4.6A shows a control cell with healthy *Giardia* trophozoites displaying normal shape and size, while Fig. 4.6B shows the cells treated with plant extract, resulting in shrinkage and rupture of the cell membrane of the trophozoites. Although the plant extract can alter the morphology of *Giardia* trophozoites, the mechanism of action of this drug is not yet well understood, and further research is necessary.

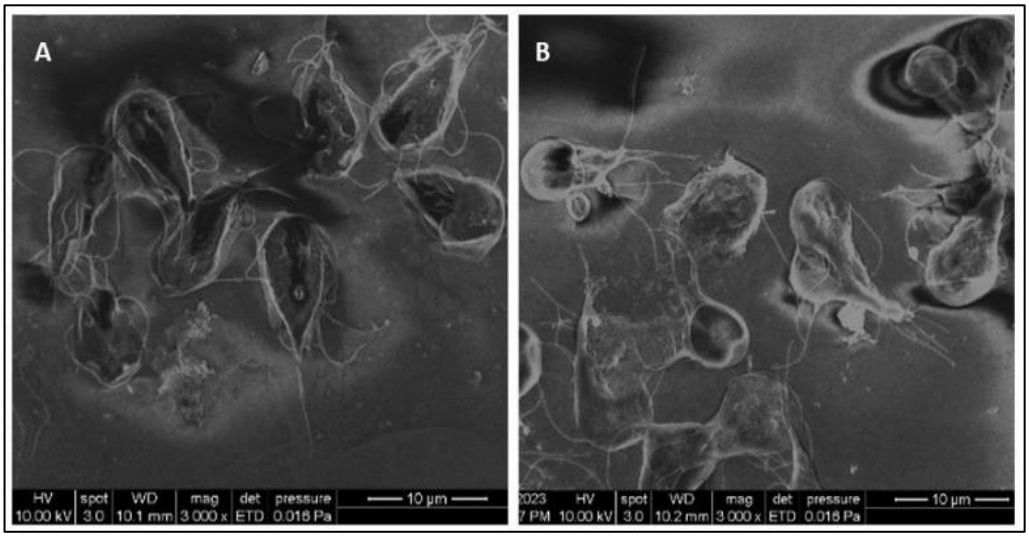


Figure 4.6: The figure shows a Scanning Electron Microscopy (SEM) image of *Giardia* trophozoites after being treated with the IC_{50} concentration of *Ap* extract for 24 hours. Panel A shows untreated cells, where normal *Giardia* trophozoites are observed, and no changes in morphology are evident. In contrast, Panel B shows extract-treated cells where trophozoite morphology has changed, and cell membrane rupture is also observed.

4.3.6. ROS generation

We investigated the effect of the plant extract on intracellular reactive oxygen species (ROS) generation in trophozoites, which has been associated with various stress conditions and its potential role in cell cycle arrest or cellular apoptosis. Trophozoites were treated with ethanolic extract at IC_{50} concentration. The results revealed a notable increase in ROS generation with longer exposure to the extract, as indicated by the progressive rise in DCF fluorescence levels (Fig. 4.7C). It is important to note that in the negative control group, there was no significant ROS generation observed. Conversely, the positive control group treated with H2O2 showed considerable ROS generation, validating the sensitivity of our ROS detection method.

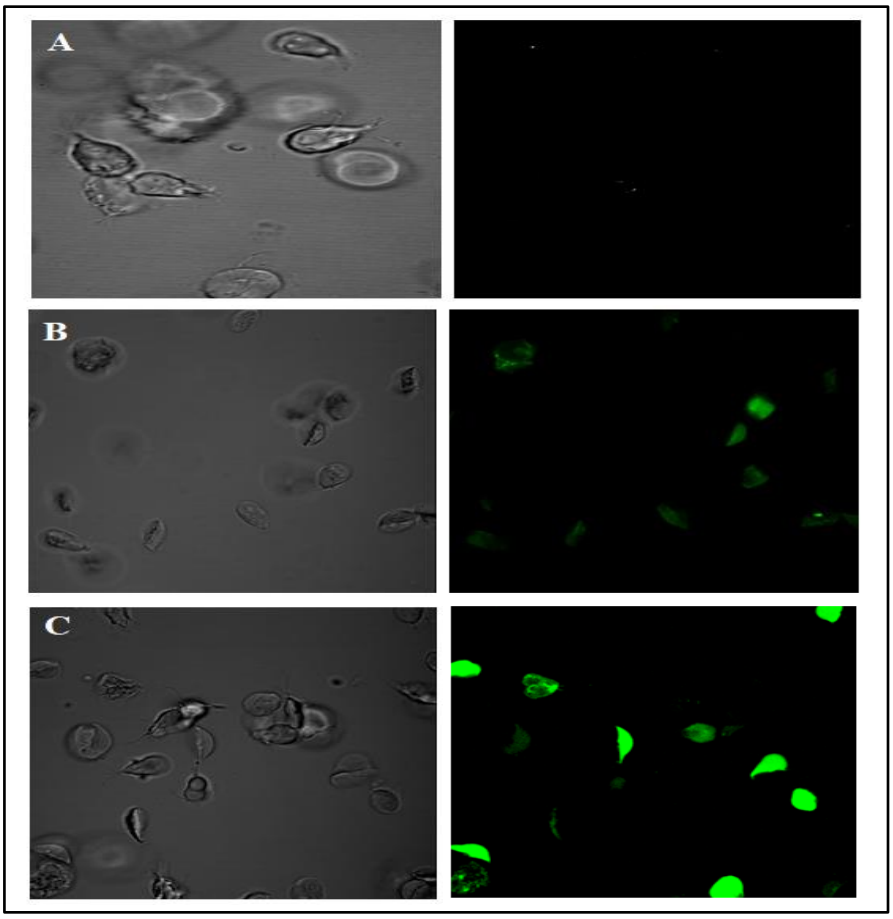


Figure 4.7: Reactive Oxygen Species (ROS) generation in *Giardia* trophozoites. A) Control group no ROS generation is observed. B) ROS generation is observed after incubated with H2O2. C) ROS generation is enhanced following incubated with *Ap* extract.

4.4. Discussion

Nitro-imidazole drugs, including tinidazole, metronidazole, and ornidazole, are commonly prescribed in clinical practice to treat giardiasis. Typically, these drugs can achieve a cure rate of around 90% within a treatment period of 5-7 days, and similar efficacy has been observed with single-dose treatment using tinidazole or ornidazole (Gardner & Hill 2001). However, the use of these drugs is associated with significant drawbacks, such as broad side effects, treatment failures, and increasing drug resistance. Furthermore, all of these drugs are known to be carcinogenic and expensive, which presents additional challenges in their use for giardiasis treatment.

The extract of *A. paniculata* has been traditionally used in Indian medicine to treat liver disorders and has been found to have antipyretic properties. Its major phytoconstituents, andrographolides, have been shown to inhibit hepatic cytochrome P450 enzymes in rats and humans (Pekthong et al. 2008). They have also been found to inhibit two enzymes associated with type 2 diabetes namely alpha-glucosidase and alpha-amylase. In the search for new compounds to treat *Giardia*, exploring plant-derived drugs is a valuable area of study. In the

present study, we investigated the potential antigiardial activity of the ethanolic crude extract obtained from *A. paniculate* on *G. lamblia*, the causative agent of giardiasis. Natural products have been an important source for the development of new drugs for many centuries. Although there have been reports on the antimicrobial effects of *A. paniculate* in other studies (Hossain et al., 2021), its antigiardial effects have not yet been explored. Only a limited number of studies have examined the pharmacological activities of specific compounds derived from this plant. Herbal medicines offer a significant advantage over chemical medicines as they contain a combination of different biological compounds with varied mechanisms of action, resulting in lower risks of developing resistance. According to the report WHO, 2005, plant-derived medicines and herbs have been extensively used to treat various diseases, including infectious diseases, cardiovascular diseases, gastrointestinal disorders, diabetes, and cancer, through local or regional healing practices in both developed and developing countries. These medicines are preferred due to minimal or no industrial processing and side effects.

Alnomasy et al. 2021 reported the pharmacological effects of 48 plant species were evaluated for their antigiardia properties. The study found that the majority of the medicinal plants used in the assessment belonged to the Lamiaceae family, followed by Asteraceae and Apiaceae. These plant families have been shown to possess high amounts of phenolic compounds, flavonoids, terpenoids, and other bioactive compounds, which exhibit a broad range of biological activities, including antimicrobial effects (Mishra et al. 2009). In the studies reviewed, IC_{50} values varied between 0.1 μg/ml and 33.8mg/ml for different plant extracts, with most presenting IC_{50} values over 100μg/ml. We examined the potential antigiardial properties of the ethanolic extract of *A. paniculata* by evaluating its effect on the viability of *Giardia* trophozoites at 12h, 24h, and 48h. We observed that the extract inhibited the growth and adhesion, and altered the morphology, DNA damage, cell cycle and ROS generation of the parasite. Our present data show that compared to other plant extracts, *A. paniculata* demonstrates higher efficacy than most of the other plant species evaluated. Moreover, previous studies have reported that the extract did not show any cytotoxicity on the intestinal cell line.

Scanning electron microscopy images revealed that the extract caused the trophozoites to shrink membrane rupture and display notable alterations in morphology. The adhesion assays revealed that concentrations greater than 50μg/ml inhibited over 50% of trophozoite attachment to glass surfaces, suggesting that factors beyond microscopic observations, such as metabolic or morphological changes, may have influenced their ability to adhere. The study found that

the *A. paiculata* plant extract caused damage to the DNA of the trophozoites, which was identified as the primary mechanism for the cytotoxic effect of the extract. Previous research, including a study by Ordoñez-Quiroz et al. in 2018, has also demonstrated in vitro DNA damage in *Giardia* using drugs such as metronidazole. The treatment of *Giardia* with this extract also results in DNA damage comparable to that caused by commonly used antigiardia drugs. When DNA damage occurs, it can lead to the activation of cellular signalling pathways that trigger the production of ROS (Xu et al. 2019). ROS are highly reactive molecules that can cause further damage to cellular components including DNA, proteins, and lipids. The accumulation of ROS can lead to oxidative stress, which can ultimately result in cell death through apoptosis. Although *Giardia* lacks conventional mitochondria and does not have a well-established apoptosis pathway, studies have shown that under oxidative stress, apoptosis-like programmed cell death can occur in *G. lamblia* (Ghosh et al. 2009). Therefore, it is possible that the treatment with this plant extract may induce apoptosis-like programmed cell death in *Giardia*.

An important fact to note is that this plant is abundantly found in the Indian subcontinent. Our current in vitro research has shown promising outcomes for utilizing the plant extract as a drug ingredient against giardiasis, although further research is required to confirm its in vivo effectiveness and safety for human use. It should be remembered that plant extracts used in phytotherapy are frequently inadequately standardized and regulated. This can result in variations in the concentration of their active compounds due to factors like plant genetics, climate, soil quality, harvesting time, and extraction methods.

4.5. Conclusion:

In conclusion, *Ap* extract shows potent inhibitory effects on *Giardia* trophozoites at various doses and times. It induces excessive ROS production, causing oxidative stress that damages DNA and arrests cell cycle regulation, leading to cell death. In future studies, identifying the active compound in this plant responsible for the antigiardia property is important. These findings suggest potential targets for future research and drug development against *Giardia.*

CHAPTER-V

ANDROGRAPHOLIDE INDUCED CYTOTOXICITY AND CELL CYCLE ARREST IN *Giardia* TROPHOZOITES

Andrographolide induced cytotoxicity and cell cycle arrest in *Giardia* trophozoites

5.1 Background

In the previous chapter, we demonstrated the various properties of the plant extract against *Giardia*. However, the specific active compound has not yet been identified. It is now essential to explore and identify the main active compound responsible for the plant's antigiardial properties.

Giardia lamblia is one of the most frequent protozoan parasites that cause giardiasis worldwide. According to WHO reports annually 280 million people have been infected globally (Carter et al. 2018). Giardiasis is becoming an increasingly prevalent issue, particularly in developing country, as it continues to emerge and spread within human populations. In developed countries, the prevalence rate of giardiasis is between 2-5%, while in developing countries ranges from 20-30% (Lalle et al. 2018). The disease is typically transmitted indirectly through the consumption of food or water that has been contaminated with cysts or through person-to-person contact, especially among individuals living in unhygienic conditions. Although giardiasis can affect individuals of all ages and display symptoms, it tends to affect children more frequently than adults (Belkessa et al. 2021). Giardiasis can either be asymptomatic or result in symptoms such as diarrhoea, vomiting, flatulence, anorexia, and crampy abdominal pain during its acute phase (Escobedo et al. 2018). In addition to gastrointestinal symptoms, it may also lead to extra-intestinal manifestations such as fever, maculopapular rash, pulmonary infiltrate, lymphadenopathy, polyarthritis, urticaria and growth retardation. Metronidazole is considered the first-line treatment for giardiasis, but alternative medications like albendazole, furazolidone and tinidazole are also used (Beer et al. 2017). Adverse effects of common drugs results headaches, dizziness, metallic taste, low efficacy and treatment failure have been reported as potential issues that cannot be overlooked (Starrs & Yenigun, 2021). The development of resistance to chemotherapeutic drugs among parasites underscores the critical importance of exploring alternative therapeutic approaches. One such approach involves investigating natural plant products as potential novel anti-parasitic agents (Anthony et al. 2005).

Andrographis paniculata (Burm. F.) Nees, a member of the Acanthaceae family, is a plant that is commonly found in several Asian countries including China, India, Thailand, and Sri Lanka

(Hossain et al. 2014). It has a long history of medicinal use in both Indian and Oriental medicine. Ancient Ayurvedic texts have highlighted its potential as an herb for addressing neoplasms and have documented at least 26 Ayurvedic formulations that incorporate it in the treatment of liver disorders (Okhuarobo et al. 2014). The plant, commonly referred to as the "king of bitters," is widely recognized for its highly bitter characteristics and has been traditionally employed as a treatment for various ailments, including the common fever, cold, dysentery, tonsillitis, liver ailments, diarrhoea, inflammation, herpes, and many others (Hossain et al. 2014). The plant contains various active compounds, such as diterpene lactones, flavonoids, and polyphenols, as reported in studies (Okhuarobo et al. 2014). Andrographolide (ADG), a diterpenoid lactone with the chemical formula $C_{20}H_{30}O_5$, serves as the primary compound responsible for the plant's therapeutic properties (Chao and Lin., 2010). It is primarily concentrated in the leaves of the plant and can be readily isolated from plant crude extracts as a crystalline solid (Varma et al. 2011). ADG demonstrates an extremely broad spectrum of biological activities. Recent reports suggest that it possesses anti-tumour, cardio-protective, anti-HIV, antioxidant, anti-inflammatory, immunomodulatory, antibacterial, cytotoxic, neuroprotective, and hepatoprotective properties (Li et al. 2022). Furthermore, it has been shown to exhibit antimicrobial activity against bacteria and viruses.

5.2. Methodology

5.2.1 Parasite Culture

In all experiments, *G. lamblia* trophozoites (ATCC 30888, Portland 1 strain) were used. *Giardia* trophozoites axenic culture were maintained in TYIS-33 medium at 37°C. The media was supplement with penicillin streptomycin antibiotic and enriched with adult bovine serum 10% (Diamond et al. 1978; Raj et al. 2014). The subculture was maintained every 48 hours and only the trophozoites from the logarithmic phase were used for all experiment.

5.2.2 Intestinal Cell Culture

Intestinal cells 407 (INT-407), originating from human embryonic tissue of the jejunum and ileum, were cultured in 25 cm2 flasks at 37°C. DMEM (Dulbecco's modified Eagle's media)

was used for culturing the cells, and it was enriched with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin.

5.2.3 Andrographolide

The active compound Andrographolide is derived from the *Andrographis paniculata* plant. We have purchased this compound from Sigma Aldrich (USA purity ≥ 98% 365645).

5.2.4 In vitro cytotoxicity assay

We used trypan blue to assess the cytotoxic effect of ADG on *Giardia* trophozoites. ADG was diluted to a concentration lower than 0.1% with DMSO in the final solution. In this assay, following treatment with different concentrations of ADG (3-20 µM), tubes were cooled for 10 minutes and then centrifuged at 250xg for 10 minutes at 4°C. The parasites were washed with PBS and subsequently incubated in a 0.4% trypan blue solution at room temperature for 5 minutes. Finally, the parasites were counted under a microscope using a hemocytometer (Carvalho et al. 2014). We conducted the experiment by exposing the parasites to various doses and times of ADG and assessing the mortality. After 24 hours of treatment, the IC_{50} value for trophozoites was determined by using nonlinear regression online ICEstimator software (http://www.antimalarial-icestimator.net/runregression1.2.htm).

5.2.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is employed to observe and analyse morphological alterations following the administration of ADG. The ADG treated trophozoites cells were washed with PBS (pH 7.2) buffer by centrifugation at 1500rpm for 10 minutes. The cells were then fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. After fixation, the samples were dehydrated through a serial dilution using alcohol solutions with increasing concentrations, ranging from 30%, 50%, 70%, 90% and 100% ethanol. To remove any remaining alcohol from the sample without causing structural damage, 2 ml of hexamethyl disilaxane were added and incubated for 10 minutes. After adding 1 ml hexamethyl disilazane the samples were incubated for an additional hour. Finally, the samples were kept in a fume hood overnight for proper safety. To enable SEM imaging, the sample was mounted onto a stub

using conductive adhesive and coated with a thin layer of gold using evaporation techniques. This coating process enhances the conductivity of the sample, which is crucial for SEM imaging. The prepared samples were imaged using SEM.

5.2.6 The Nuclei Staining

DAPI (4', 6-diamidino-2-phenylindole) staining was employed to investigate DNA damage in trophozoites following treatment with ADG. The trophozoite culture treated with ADG was harvested and then subjected to centrifugation at a lower speed (1500 rpm) for 10 minutes to pellet the parasites. Gently removed the supernatant and pellets were washed by resuspending with 1X PBS (pH 7.2). Following fixation with 4% formaldehyde, the cells were incubated with a blocking buffer that included 0.1% Triton X-100 for a duration of 1 hour. DAPI stock solution was added directly to the resuspended pellet at a final concentration of 1-10µg/ml and gently mixed to ensure uniform distribution. The samples were incubated in dark for 30 minutes at room temperature to allow DAPI to penetrate the *Giardia* trophozoites nuclei. After the incubation period, the sample was centrifuged at a low speed (1500 rpm) for 10 minutes to pellet the stained parasites. The pellet was washed by resuspending with PBS and the centrifugation step was repeated at least three times to remove excess DAPI or any non-specifically bound dye. The coverslip-mounted samples were prepared on glass slides and the slides were then imaged using a Confocal Microscope (Zeiss LSM 710).

5.2.7 Cell Cycle Study

To explore the impact of ADG on *Giardia* cell cycle, we utilized a PI/RNase solution (Invitrogen) in flow cytometry analysis to detect potential cell cycle arrest. *Giardia* trophozoite cells were treated with ADG, harvested and then washing with 1X PBS (pH 7.2) through centrifugation at 1500 rpm for 10 minutes at 4°C. The resulting pellet was dissolved in 70% ethanol and fixed overnight at 4°C. During fixation, 100µl of 70% ethanol was added repeatedly while vortexing to prevent cell clumping. After centrifugation 1500 rpm for 10 minutes, 400 µl of PI/RNase staining solution was added and incubated for 1 hour at 37°C. Finally, the cell cycle was analyzed by flow cytometer (BD FACSAria^{TM} II).

5.2.8 Determination of intracellular ROS generation

We performed a DCFDA assay to observe ROS (Reactive Oxygen Species) generation in *Giardia* trophozoites after treatment with ADG. For each set, 4×10^6 *Giardia* trophozoites were collected and suspended in PBS. Following this, 2 µl of DCFDA (2'-7'-Dichlorodihydrofluorescein diacetate) (Invitrogen) from 100 µM stock solution was added to each case. Subsequently, all the sets were incubated at 37 ℃ for 15 minutes in absence of light. After the incubation, the samples underwent three washes PBS (pH 7.2), and finally, slides were prepared for confocal examination. We used a Confocal Microscope (Zeiss LSM 710) for data generation with an emission wavelength of 530 nm and an excitation wavelength of 488 nm.

5.3 Results

5.3.1 Cytotoxic effect

ADG demonstrated a potent, dose-dependent inhibition of parasites compared to the control. After 24 hours, the IC_{50} value for ADG is 4.99 µM, which is almost similar to metronidazole (Argüello-García et al. 2018). Additionally, it was observed that the 80 µM ADG concentration did not show any cytotoxic effect on INT-407, normal human intestinal cells. The results showed (Fig. 5.1) a significant reduction in trophozoite as the concentration of ADG increased, indicating a dose and time-dependent effect. With an ADG concentration of 3µM, the average percentage of cell death reached 36.35%. Furthermore, the study revealed that increasing the ADG concentration to 6µM resulted death rate of 44.1%, while a concentration of 9µM cell death rate of 62.76% and 15 µM contains 76.47% of cell death. Notably, 91.18% of the cells death occur even at the highest tested concentration of ADG 20µM for 24 hours.

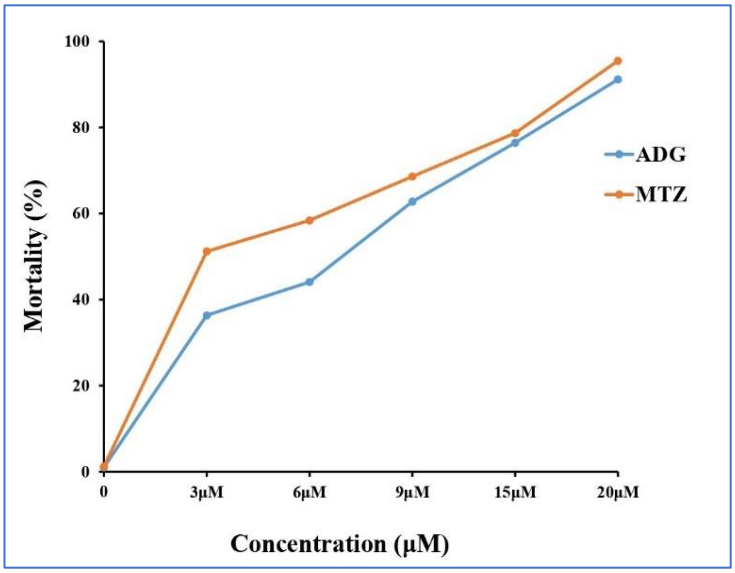


Figure 5.1: The assessment of cell death activity of ADG compared to metronidazole. *Giardia lamblia* trophozoites were treated with ADG and metronidazole at various concentrations (3–20 µM) 0.1% DMSO control for 24 hours of incubation. The dose dependent inhibitory effects of ADG were evaluated using trypan blue assays. The experiment was conducted in triplicate and the IC_{50} growth inhibition of ADG against trophozoites was 4.99 µM after 24 hours of incubation.

The study examined the mortality rate of *Giardia* trophozoites when exposed to ADG IC_{50} concentration the trophozoites were monitored at different time intervals to assess the impact of the treatment. Initially, after 4 hours of treatment, no significant effect on trophozoites was observed. However, in Fig. 5. 2 after 12 hours of treatment, the activity of the compound noticeably increased, resulting in a higher frequency of trophozoite death over time. As the incubation period was prolonged to 48 hours and 72 hours, the cell death reached its maximum. Particularly, at the 72-hour time point, the ADG exhibited its highest level of activity, causing 84.0% cell death.

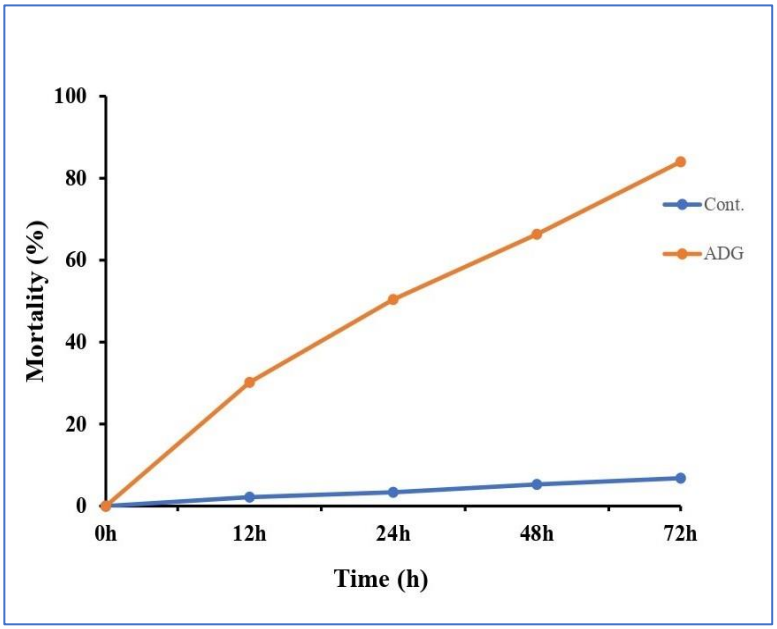


Figure 5.2: The effect of ADG on *Giardia* trophozoites. Trophozoites were treated with IC_{50} concentrations of ADG for various time points (0, 12, 24, 48, 72 hours). The experiment was conducted in triplicate and the results were evaluated using trypan blue assay.

5.3.2 Morphology Changes

We observed that the administration of ADG could induce changes in the shape and size of *Giardia* trophozoites. Particularly, cells treated with IC_{50} concentration of ADG exhibited significant alterations. In Figure 5.3A, a control cell containing healthy *Giardia* trophozoites is depicted, showing their normal shape and size, with visible flagella. In contrast, Figure 5.3B illustrates the impact of ADG treatment on the cells, leading to shrinkage and the rupture of the trophozoites cell membrane.

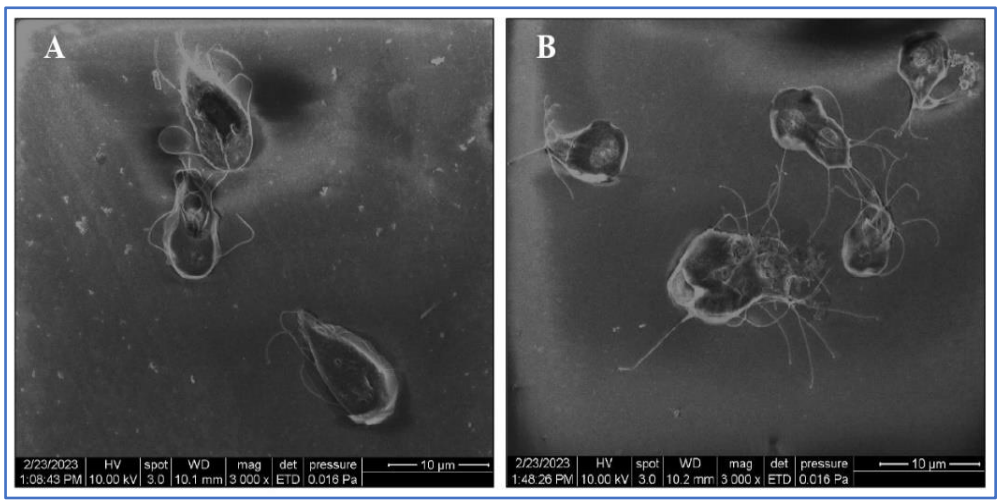


Figure 5.3: The figure shows a Scanning Electron Microscopy (SEM) image of *Giardia lamblia* trophozoites after being treated with the IC_{50} concentration of ADG for 24 hours. Panel A shows untreated cells, where normal *Giardia* trophozoites are observed, and no changes in morphology are evident. In contrast, Panel B shows ADG-treated cells where trophozoite morphology has changed, and cell membrane rupture is observed.

5.3.3 DNA Damages

The study investigated the impact of ADG on *Giardia* trophozoites and its potential to induce DNA damage. The results in Fig. 5.4B revealed that treatment with ADG at IC_{50} concentration led to observable as indicated by a positive signal nucleus damage in *Giardia* trophozoites. In contrast, non-treated control trophozoites showed no evidence of nucleus damage Fig. 5.4A. Furthermore, DNA degradation is a characteristic feature associated with apoptotic cell death.

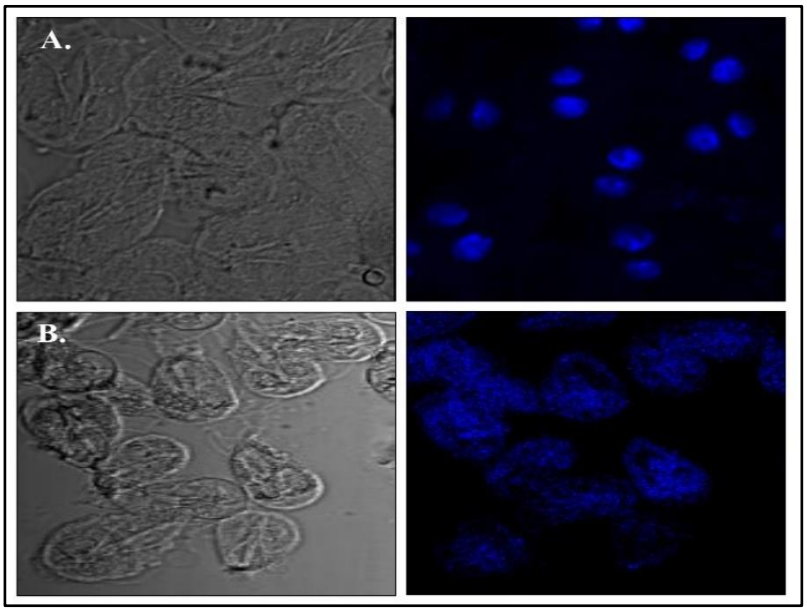


Figure 5.4: The figure shows the results of a DNA degradation analysis (A-B). In control living cells where no DNA degradation was observed (A). However, there was significant DNA damage observed after being treated with ADG (B).

5.3.4 Cell Cycle Arrest

ADG treatment of trophozoite cells resulted in significant cell cycle arrest, as observed from the PI binding. The fluorescence intensity detected in apoptotic cells, which corresponds to fragmented DNA at G0/G1 phases of cell cycle. This region is commonly referred to as the sub G0/G1 peak. The sub G0/G1 region exhibited a marked and significant increase the amount of

apoptotic cells as the duration of ADG treatment. Quantitative analysis of our results (Fig. 5.5) revealed that the S phase, control cells showed approximately 68% at the start, 50.4% at 12 hours, 36.4% at 24 hours and 6.3% at 48 hours. In contrast, in the G0/G1 phase, control cells displayed a distribution of 14.8% at the beginning, 38.8% at 12 hours, 44.9% at 24 hours and 20.7% at 48 hours. Notably, at the 48-hour time point, a significant population of cells, comprising 69.5%, had arrested in the Sub G0 phase. These findings indicate a significant cell cycle arrest induced by ADG treatment.

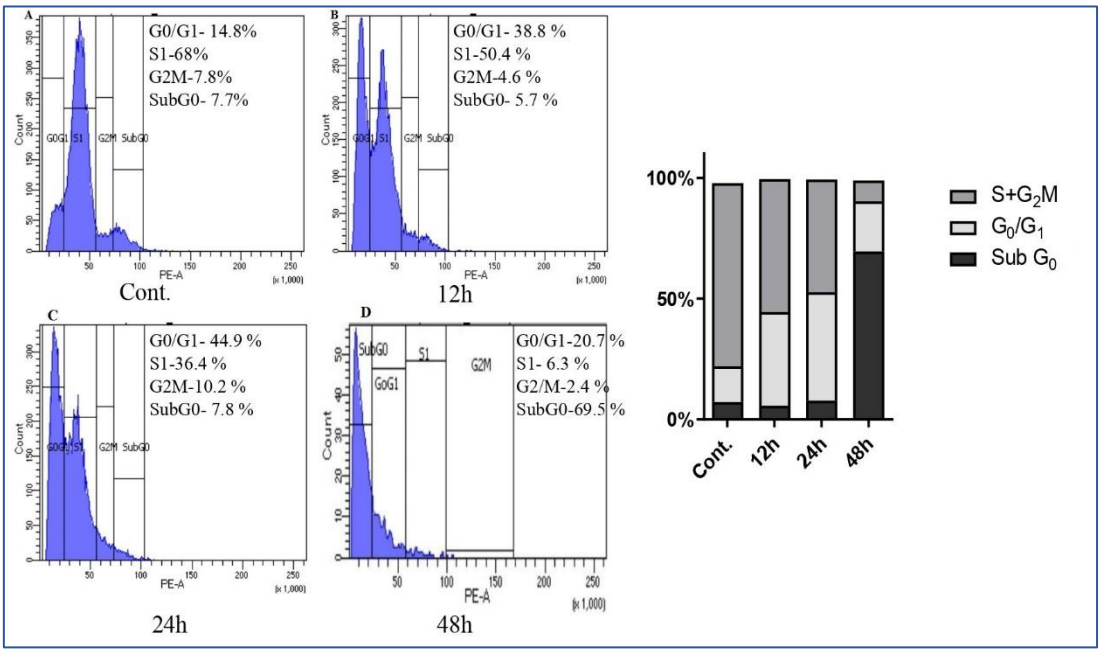


Figure 5.5: The effect of ADG treatment on the cell cycle of *Giardia* trophozoites using flow cytometry analysis: Control cells showing the trophozoites normal DNA content in S phase (A). Cells were exposed to the IC_{50} concentration of the ADG and incubated for different time points: 12h (B), 24h (C), and 48h (D). The ADG treatment caused cell cycle arrest, leading to a significant arrested the cells in G0/G1 and subG0 phase.

5.3.5 ROS generation

The study shown the effects of ADG treatment on oxidative stress generation and ROS production. We observed that as the concentration of ADG increased, there was an escalation in the rate of trophozoite death. Through time kinetics and dose kinetics are analysed, we found that after 24 hours of incubation, the viability of *Giardia* trophozoites significantly decreased, leading to their detachment from the glass tubes surface. In order to evaluate the production of reactive oxygen intermediates within the *Giardia* trophozoites. We observed that trophozoites treated with ADG exhibited significantly higher fluorescence intensity compared to non-treated

trophozoites (Fig. 5.6A & 5.6B). The result indicate an absence of ROS generation in untreated cells, whereas a notable increase in ROS generation is observed in cells treated with ADG.

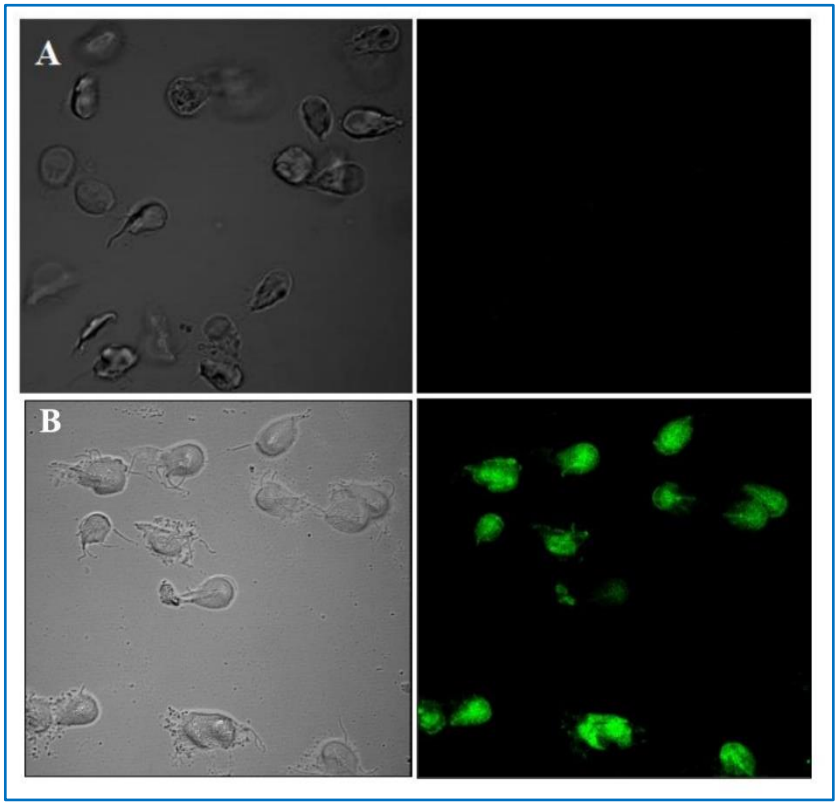


Figure 5.6: ROS generation in *Giardia* trophozoites. (A) Control group no ROS generation is observed. (B) ROS generation is enhanced following incubated with ADG.

5.4 Discussion

The anti-cancer and immuno-stimulatory properties of bioactive compounds derived from *A. paniculata* have gained significant attention in recent years. Numerous reports have focused on the ability of ADG, one of these compounds to induce cellular apoptosis in various cell lines (Kumar et al. 2004; Sukardiman et al. 2007). Notably, a recent study explored the cytotoxic effects of ADG on human oral epidermoid carcinoma cell Meng-1 (OEC-M1), revealing its impact on cellular viability, morphology and migration (Liao et al. 2022). Moreover, in this study we examined the in vitro inhibitory effects of ADG on the proliferation and cell death of *Giardia* trophozoites, demonstrating a time-dependent and dose-dependent relationship. The findings reveal that ADG exerts a strong inhibitory effect on the survival of trophozoites with

an IC_{50} concentration observed after 24 hours of treatment. Moreover, it induces cellular apoptosis-like cell death, characterized by alterations in cellular morphology, DNA damages and the generation of ROS.

In an earlier study, the effects of ADG on PC-3 prostate carcinoma cells were examined revealing significant morphological and biochemical changes (Kim et al., 2005). ADG has also been found to possess cytotoxic activity against KB cells (human epidermoid carcinoma) and P388 cells (lymphocytic leukemia). The immunostimulatory and anticancer properties of ADG have been observed across various human cancer cell lines (Kumar et al., 2004). In our research, scanning electron microscopy images showed that the ADG led to remarkable changes in morphology of the trophozoites. These structures are crucial for the attachment of protozoans to the intestinal cell surface. The study suggests that factors beyond microscopic observations, such as metabolic or morphological changes played a role in affecting the adhesion ability of the trophozoites.

ROS are molecules with high reactivity that can result in oxidative stress and damage the cellular structures. Studies have indicated that ADG has the ability to stimulate the production of ROS in different organisms, including cancer cells (Banerjee et al. 2017). Notably, in certain instances, ROS can exhibit antimicrobial properties by interfering with cellular processes and causing damage to DNA and proteins (Vatansever et al. 2013). The excessive generation of ROS and RNS can deplete intracellular antioxidant compounds, ultimately resulting in cell death (Ghosh et al. 2009). Our findings demonstrated that exposure to ADG led to the production of excessive intracellular ROS in *Giardia* trophozoites. Notably, ROS were detected, suggesting that oxidative stress induced by ADG could contribute to the cytotoxic effects of this compound in *Giardia* trophozoites. In future investigations it is crucial to identify the specific ROS involved and elucidate the mechanisms responsible for their formation. Recent studies have revealed the ability of certain drugs such as metronidazole, to induce damage to DNA in *Giardia* through in vitro experiments (Uzlikova & Nohynkova, 2015). Similarly, other redox-active drugs like benzimidazole and hydroxymethylnitrofurazone have been reported to primarily affect DNA, a characteristic often associated with necrosis (Davies et al. 2014). When *Giardia* trophozoites were exposed to ADG, DNA degradation was observed, indicating that DNA is the molecule most susceptible to damage. Specifically, treatment with ADG resulted in cellular damage and oxidative stress in the parasites. While these observations suggest that ADG acts as a DNA-damaging agent, it is important to note that the cytotoxic effects of ADG treatment are multifaceted, as indicated by other research

findings. Furthermore, we have demonstrated that the treatment of *Giardia* trophozoites with ADG leads to a rapid decrease in cell adherence, ultimately leading to cell death.

The DNA damage is a molecular occurrence that is strongly linked to the arrest of the cell cycle and the initiation of cell death. In this particular investigation, the administration of ADG led to a reduction of cells in the S/G2M phase. This decrease can be attributed to the interference of ADG with the normal progression of the cell cycle, specifically inhibiting the transition from the S phase to the G2/M phase. Therefore, mitosis is arrested and the cell cycle comes to a halt at the S phase, ultimately triggering cellular apoptosis (Ma et al. 2012; Hung et al. 1996). Our research findings align with previous studies that have demonstrated an apoptotic-like process in *Giardia* when exposed to oxidative stress inducers and metronidazole (Bagchi et al. 2012). Furthermore, multiple studies have provided evidence that ADG is capable of inducing cell cycle arrest predominantly at the G0/G1 stage in a wide range of cancer cells (Geethangili et al. 2008). However, our study presents a novel finding by demonstrating cell cycle arrest at the sub G0/G1 phase specifically in *Giardia* trophozoites upon treatment with ADG. Further research needs to better understand the underlying mechanisms responsible for cell cycle arrest in ADG-treated trophozoites.

5.5 Conclusion

ADG has demonstrated potent inhibitory activity against *Giardia* trophozoites at various doses and time intervals. It also triggers the generation of ROS, leading to oxidative stress. The oxidative stress primarily damages DNA, leading to double-strand breaks and inhibition of cell cycle regulation, ultimately resulting in cell death. These findings provide potential targets for future research and drug development against *Giardia*, considering the parasites exposure to oxidative stress in the gut. Targeting excessive ROS generation could be a promising strategy for developing new antigiardia drugs.

CHAPTER-VI

CLONING AND EXPRESSION OF CATHEPSIN-B GENE OF *Giardia.*

Cloning and expression of Cathepsin-B gene of *Giardia*

6.1. Background

We demonstrated various antigiardial properties of the plant's active compound (andrographolide) in the previous chapter. Currently, it is necessary to study the effect of andrographolide on *Giardia* pathogenicity. The aim of this chapter is to focus on cloning and antibody generation of the Cathepsin-B gene for future proteomics studies on *Giardia* pathogenesis.

Giardia lamblia is a protozoan parasite that resides in the upper small intestines of mammals and plays a substantial role in causing waterborne diarrhoea worldwide (Ankarklev et al. 2010; Halliez et al. 2013). The parasite exhibits eight distinct genotypes or assemblages labeled from A to H, with assemblages A and B specifically affecting humans (Certad et al. 2017). The infectious form of *Giardia* is the cyst, which is acquired through the oral-fecal route. Upon reaching the duodenum and jejunum, the cyst ruptures, releasing excyzoites that promptly transform into trophozoites (Ankarklev et al. 2010). These trophozoites attach to the apical surface of intestinal epithelial cells (IECs) using an adhesive disc (Dawson et al. 2010). The intimate contact and ensuing interactions initiate a series of pathophysiological alterations, ultimately resulting in symptoms such as diarrhoea, malabsorption, and weight loss (Einarsson et al. 2016). The evident manifestation of these outcomes is particularly pronounced in individuals with compromised immune systems, the elderly and young children in developing regions (Mmbaga et al. 2017). Emerging data strongly indicate the involvement of giardial cysteine proteases (CPs) in the induction of disease and the pathogenic processes (Cotton et al. 2015). An observed escalation in CP secretion has been documented during in vitro host-parasite interactions (Rodriguez-Fuentes et al. 2006). Additionally, studies have demonstrated that CPs have the capacity to disrupt cellular junctions, thereby compromising the integrity of the intestinal epithelial barrier (IEB) (Chin et al. 2002).

Newly discovered evidence suggests that cysteine protease (CP) actions derived from *Giardia* can initiate the cleavage of the microvillus protein villin (Bhargava et al. 2015). Additionally, these CPs can cleave the chemokine IL-8, leading to a reduction in inflammation (Cotton et al. 2014). Furthermore, they influence the normal bacterial flora and biofilm formation (Gerbaba et al. 2015; Beatty et al. 2017) and exhibit inhibitory effects on the growth of intestinal bacterial

pathogens (Manko et al. 2017). Collectively, these studies underscore the significant role played by CP activities in the interactions between the host and *Giardia*. Nevertheless, the examination of CPs in *Giardia*'s pathogenicity has predominantly relied on cell extracts or CP inhibitors. However, there is a need for further investigations to elucidate the specific roles of individual CPs from *Giardia* in the mechanisms underlying the disease. In the *Giardia* WB genome, CPs constitute the most prevalent types of proteases, with 26 genes, including 4 cathepsin C-like, 9 cathepsin B-like, and 13 cathepsin K/L-like genes (Morrison et al. 2007; DuBois et al. 2008). The cathepsin B-like proteases emerge as the most prominently expressed among cathepsins, with many experiencing upregulation during differentiation and *Giardia*-intestinal epithelial cell (IEC) interactions in vitro (Ringqvist et al. 2011; Emery et al. 2016). Certain specific CPs have been implicated in distinct processes, including excystation, with CP2 (CP14019), CP1 (CP10217), and CP3 (CP16779) have been suggested contributors (Ward et al. 1997). Additionally, CP14109 has been linked to encystation, while CP14019 plays a role in the degradation of endocytosed proteins (DuBois et al. 2008).

In this study, our objective was to cloning of cathepsin cysteine proteases (CP14019). Within the realm of cathepsin cysteine proteases, a distinctive feature lies in their catalytic dyad, comprising active-site cysteine (Cys) and histidine (His) residues. These proteases fall under the classification of clan CA cysteine proteases, which, in turn, branches into various superfamilies, including those resembling cathepsin B (catB, cathepsin L) (DuBois et al. 2006). Cathepsin B proteases are distinguished by an additional 20-amino-acid insertion known as the occluding loop, housing two characteristic His residues. This insertion enhances their functionality as either endo- or exopeptidases (Halliez et al. 2016). Within the *G. duodenalis* genome, numerous genes encode cathepsin cysteine proteases, the majority of which lack well-defined functions (Roxstrom et al. 2005; Dubourg et al. 2018). Nevertheless, in response to intestinal epithelial cells (IECs), several cathepsin proteases in *G. duodenalis* are observed to be upregulated (Mach et al. 1994).

6.2. Methodology

6.2.1. Cell Culture

G. lamblia trophozoites strain (ATCC 50803) were cultured in TYI-S-33 medium supplemented with penicillin, streptomycin, and 10% adult bovine serum at 37°C. The medium's pH was adjusted to 6.8 during filter sterilization using a 0.22µm filter within 10 mL glass tubes. After approximately 2 days, when reaching the logarithmic phase, the cultures were

transferred to 15 mL centrifuge tubes. Subsequently, trophozoites were harvested by centrifugation at 2000 rpm for 10 minutes at 4°C.

6.2.3. RNA Isolation

To extract RNA from approximately $2x10^8$ *Giardia* trophozoites cells in their logarithmic growth phase and 1 ml of Trizol reagent was introduced to facilitate cell disruption. The disruption process involved rapid pelleting for 5 minutes, followed by a 5-minute incubation period at room temperature, and further RNA isolation involved adding 0.2 mL of chloroform for every 1 ml of Trizol used. The tubes were then securely capped, and manual agitation by hand for a duration of 15 seconds was performed. These sequential steps were integral to the initial phase of RNA extraction from the *Giardia* cells. After the initial processing steps for RNA extraction from the *Giardia* cells, the samples were allowed to incubate at room temperature for 5 minutes. Following this incubation, a centrifugation step was carried out at 12,000g for a duration of 20 minutes, maintaining a temperature range of 2-8°C. This centrifugation process led to the separation of the contents into three distinct phases: a lower red phenol chloroform phase, an interphase, and an upper aqueous phase that was clear and colourless. To advance the RNA extraction process, the upper aqueous phase was carefully transferred to a fresh tube. For RNA precipitation, 0.5 mL of isopropanol was introduced and mixed with the aqueous phase. Subsequently, the mixture was incubated within the temperature range of 15-30°C for a period of 10 minutes. These steps played a pivotal role in further isolating and concentrating the RNA from the samples. After the RNA precipitation step, the sample underwent further processing. Specifically, it was subjected to centrifugation at 12,000g for a duration of 10 minutes within a temperature range of 2-8°C. This centrifugation process resulted in the formation of a gel-like RNA pellet at the bottom of the tube.

The RNA pellet was subsequently subjected to a single wash using 75% ethanol. Following this ethanol wash, the sample was mixed and subjected to centrifugation once more, this time at 7,500g for a duration of 5 minutes at a temperature range of 2-8°C. This additional centrifugation step was aimed at further purifying the RNA. Following these processing steps, the RNA pellet was carefully air-dried, ensuring it retained slight moisture to prevent interference with solubility. Finally, to prepare the RNA for downstream applications, it was dissolved in DEPC (Diethyl pyrocarbonate) water. The dissolution process occurred at a temperature between 55-60°C for a duration of 5 minutes, preserving the RNA's solubility and integrity.

6.2.4. cDNA Preparation

To prepare the reaction mixture, combine 1 µl of oligo(dT)12-18 (500 µg/ml) and 1 µl of a dNTP mix (10 mM each) with 5-10 ng of total RNA or 1-2 ng of mRNA in a total volume of 20 µl. Adjust the total volume to 12 µl with sterile water. Heat the mixture to 65°C for 5 minutes and then rapidly cool it on ice. After a quick centrifugation, gather the tube's contents and incorporate them into the reaction mixture, or you can create a master mix and add the collected contents. To the reaction mixture obtained in the previous step, introduce 4 µl of 5X First Strand Buffer, 2 µl of 0.1 M DDT, and 1 µl of RNase inhibitor. Carefully blend the contents and let them sit at room temperature for 5 minutes. Subsequently, add 1 µl of super RT (2000 U/µl) and gently mix by pipetting up and down. Incubate the mixture within the temperature range of 42-52°C for 50 minutes. Finally, inactivate the reaction by subjecting it to heating at 70°C for 15 minutes.

6.2.5. PCR Amplification

To initiate cDNA amplification, a PCR procedure was carried out using a DNA thermal cycler. In sterile 0.5 mL PCR tubes, the essential reagents were combined as follows: 10X Buffer (5 µL), magnesium chloride (2 µL), dNTPs (3 µl), forward primer (1 µl) harboring the *Giardia* Cathepsin Forward Primer Sequence (5'-TAAGCAGAATTCATGAAGCTCTTTCTCCTC-3' - containing a Bam HI site) and reverse primer (1 µL) containing the *Giardia* Cathepsin Reverse Primer Sequence (5'-TGCTTAAAGCTTTTACTCATCGAAGAAGCC-3' - featuring a Hind III site). These tubes were then gently vortexed and briefly spun to ensure proper mixing of the chemical components. The PCR reaction mixture, totaling 50 µl in volume, comprised 3 µL of template, 0.5 µL of Ex Taq polymerase, and 35.5 µL of water. The amplification process consisted of 35 cycles, involving denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and elongation at 72°C for 45 seconds. To ensure complete denaturation of the template, the reaction mixture underwent an initial incubation at 94°C for 10 minutes, followed by a final extension step at 72°C for 5 minutes upon conclusion of the reaction. The PCR product, estimated to be around 1kb in size, was subjected to electrophoresis within a 1% agarose gel, and subsequently, the DNA band was extracted. This eluted RT-PCR product underwent end-filling using a nuclease, followed by digestion with BamHI. The resulting digested product was then ligated into the Pet vector at the XhoI and BamHI sites. To conclude the procedure, a positive clone was identified by introducing the ligation mixture into *E. coli* cells, which were then cultured on plates containing LA Kanamycin.

6.2.6. Restriction Digestion

The process of restriction digestion entails the use of specialized enzymes called Restriction endonucleases (REs) to precisely divide DNA molecules into smaller fragments. These enzymes, often likened to molecular scissors, bear names derived from the specific organisms of origin. REs possess the remarkable capability to recognize specific sequences termed restriction sites within double-stranded DNA, subsequently cleaving the DNA by disrupting each phosphate backbone of the double helix. To prepare the reaction mixture, all the necessary reagents were thawed on ice and sequentially added to a microcentrifuge tube, resulting in a total volume of 25 units. The reagents were introduced in the following order for both insert the plasmid: 2 µl of buffer, 1 µl of RE Bam HI, 1 µl of RE Hind III, and 12 µl of water (Takara BioTM Kit). The tube was gently mixed by tapping and then briefly centrifuged to ensure proper settling of the contents. Additionally, a negative control tube-lacking template DNA was also set up. The reaction was subsequently incubated at 37°C for 1 hour, followed by the inactivation of the restriction enzymes via incubation at 65°C for 10 minutes.

6.2.7. Ligation

T4 DNA Ligase is an enzyme that aids in the joining of two DNA strands by linking the 5' phosphate and 3' hydroxyl groups of adjacent nucleotides, whether they are in a cohesive or blunt-ended configuration. This versatile enzyme has the capacity to fuse RNA to a DNA or RNA strand within a double-stranded molecule but cannot connect single-stranded nucleic acids. When cloning a fragment into a plasmid vector, it is advisable to use a molar ratio of 1:1, 1:3, or 3:1 for the vector-to-insert DNA ratio. In a sterile microcentrifuge tube, a reaction mixture was prepared, comprising 100 ng of vector DNA, 17 ng of insert DNA, 1 µl of 10X ligase buffer, 0.1-1 U of T4 DNA Ligase, and nuclease-free water added to reach a final volume of 10 µl. The mixture was gently homogenized by pipetting and allowed to incubate at 16 degrees Celsius overnight. Following this incubation, it was subjected to heat inactivation at 65 degrees Celsius for 10 minutes. Subsequently, the reaction mixture was rapidly cooled on ice, and 5 µl of the ligated products were used for transformation into 50 µl of competent *E. coli* DH5 cells.

6.2.8. Preparation of Competent Cells

On the first day, *E. coli* DH5α strain was streaked onto an LBM plate without ampicillin to isolate individual colonies. The plate was then incubated at 37 degrees Celsius for an extended

period of 16-20 hours. On the second day, a sterile inoculating loop was employed to collect cells from a single colony, which were subsequently used to inoculate 50 ml of sterile 1X LBM. This culture was then grown overnight at 37 degrees Celsius in a shaker incubator for 16-20 hours. Additionally, two 250 ml flasks containing 1X LBM were placed in the incubator to equilibrate the medium's temperature.

On the third day of the procedure, several steps were carried out. Initially, 25 ml of the overnight bacterial culture was added to each of the 250 ml LBM flasks. Additionally, another 150 ml flask containing 1X LBM was placed in the incubator to adjust to the medium's temperature. The cultures were allowed to grow until they reached an optical density (OD) of 0.2 at 650 nm. Subsequently, 75 ml of equilibrated 1X LBM was added to each flask, and the incubation continued for an additional 30 minutes. Following this, the cells were pelleted in chilled, autoclaved large centrifuge bottles using the Beckman J6 centrifuge at 5000 rpm for 10 minutes. It was crucial to maintain the cells at a low temperature throughout the procedure; thus, tubes were kept on ice, and resuspension occurred in the cold room. After decanting the supernatant, the cells were resuspended in 1/4 of their original volume of ice-cold 100 mM MgCl2 and left on ice for 5 minutes. Finally, the cell suspension was transferred to pre-chilled sterile large centrifuge bottles and spun in the centrifuge for 10 minutes at 4000 rpm at 4 degrees Celsius. Continuing with the procedure, the next steps were executed on day three. Firstly, the supernatant was decanted, and the cells were resuspended in 1/20 of their original volume of ice-cold 100 mM CaCl2. This resuspension was left on ice for a duration of 20 minutes and then subjected to centrifugation at 4000 rpm for 10 minutes, following the same cooling precautions. Subsequently, the supernatant was decanted once more, and the cell pellet was resuspended in 1/100 of their original volume of a solution composed of 85% v/v 100 mM CaCl2 and 15% v/v glycerol. For each culture processed, Eppendorf tubes were pre-chilled in a dry ice ethanol bath. The competent cell aliquots were then transferred to -80˚C for storage. After 24 hours, the efficiency of transformation was assessed by using 1 ng, 10 ng, and 100 ng of an ampicillin-resistant plasmid on LBM+ Amp plates, following the transformation protocol for intact plasmids. To gauge the background level, 50 µl of cells alone were plated on an LBM+ Amp plate. The expected yield was approximately 5x10^7 colonies per microgram of supercoiled DNA.

6.2.9. Transformation of pET28a-CathB in *E. coli* BL21 (DE3)

For the transformation process, commence by thawing the competent cells on ice, simultaneously ensuring the DNA from the ligation mixture is also kept chilled. Subsequently, add 50 µl of competent cells to the DNA, gently mixing by pipetting up and down or flicking the tube 4-5 times without vortexing. Allow the mixture to put on ice for 30 minutes without any further agitation. Following this incubation, the tube to a heat shock at 42 °C for a duration of 45 seconds to 1 minute, again refraining from mixing. To the tube, add 950 µl of room temperature LB or SOC media, and place it in a 37°C environment for 60 minutes with vigorous shaking or rotation. Meanwhile, pre-warm the selection plates to 37 °C and once the incubation is complete, spread 50-100 µl of the cells and ligation mixtures onto the plates. Finally, allow them to incubate overnight at 37 °C.

6.2.10. Expression of Gene in *E.coli* BL21 (DE3)

Competent *E. coli* BL21 (DE3) cells were subjected to transformation using pET28a-CathB plasmid DNA and subsequently cultivated in Luria broth supplemented with kanamycin. The cells underwent induction with IPTG and were then centrifuged to form pellets. The total SDS-solubilized proteins were examined using a 10% SDS polyacrylamide gel, and the results were compared with the lane containing SDS-solubilized proteins from uninduced cells.

6.2.11. SDS PAGE

To summarize, 100-200 pg of sample protein, along with an appropriate volume of sample buffer, were added to the suspensions, followed by boiling for 3 minutes in a water bath. Immediate vortexing ensued, and after a brief centrifugation at 1000g, the samples were loaded into the respective wells of the gel. Electrophoresis took place in a rectangular vertical slab gel apparatus (BioRad Protean II) at 90 volts until the samples reached the top of the resolving gel. Subsequently, the voltage was increased to 120 volts. The electrophoresis process concluded when the dye front migrated to within 1-2 cm of the bottom of the gel. After the electrophoresis process, the gel was expeditiously removed from the glass plates and submerged in a tray with a polyacrylamide gel staining solution for one hour. Subsequently, it underwent destaining using a destaining solution for a period of two to three hours.

6.2.12. Kinetics of induction

The kinetics of Cathepsin B gene expression were investigated by cultivating cells in LB medium until reaching an OD600 of 0.6, followed by inducing the culture with 1.0 mM IPTG. At various time points after induction (4 hr, 6 hr, and overnight), 1 ml of the culture was pelleted. The obtained pellets were washed with normal saline and suspended in SDS-PAGE sample buffer. Subsequently, the protein samples were boiled and analyzed using a 10% SDS polyacrylamide gel.

6.2.13. Selection of optimum temperature of overexpression of Cathepsin B protein in insoluble fraction

E. coli BL21 (DE3) cells, containing the pET28a-cathB plasmid, were cultured in 50 ml of LB medium supplemented with kanamycin. The cultures were grown at temperatures of 20°C and 37°C until they reached an OD600 of 0.6, and induction was performed using 1 mM IPTG. After induction, the cells were harvested, washed with normal saline, and the cell pellet was treated with 100 µl of lysis buffer and 1 µl of lysozyme. The mixture was gently shaken for 15 minutes. Subsequently, 1 ml of DNase (1 mg/ml) was added, thoroughly mixed, and incubated at 4°C for 30 minutes. Following centrifugation at 12,000g for 15 minutes, the resulting supernatant was collected as the soluble protein fraction. Meanwhile, the pellet was reconstituted in SDS-PAGE sample buffer, containing 0.1% Triton X-100 and 8M Urea. This mixture was then boiled for 10 minutes in a water bath, followed by a rapid spin at 10,000g, and the resulting supernatant was identified as the insoluble fraction.

6.2.14. Purification of Cathepsin B

The sonicated crude insoluble fraction from the overexpression of cathepsin B was applied to a His Tag column (HiTrap Chelating: Amersham Pharmacia, USA) that had been pre-equilibrated with NiSO4 and binding buffer. The proteins not bound to the column were washed with 10 column of binding buffer, which included 5mM imidazole, followed by an additional wash with 6 eluted column buffer containing 20mM imidazole. The targeted protein was then eluted containing 250mM imidazole. Afterward, the imidazole and NaCl were removed by dialysis against binding buffer using a centricon with a pore size of 30,000 daltons. The purified protein fractions were subsequently examined through SDS-PAGE analysis.

6.2.15. Estimation of purified protein

The protein content of the purified cathepsinB was assessed using the Lowry's modified DC Bio-Rad protein estimation kit (BioRad, USA) following the manufacturer's protocol. In summary, 100 µl of standards and samples were dispensed into clean, dry test tubes. Subsequently, 500 µl of reagent A (containing reagent S) was added, and the mixture was thoroughly vortexed. Each test tube received 4.0 ml of reagent B, and the contents were promptly mixed by vortexing. The absorbance was measured after 15 minutes using the spectrophotometer. A standard solution of 1 mg/ml BSA was employed for calibration.

6.2.16. Production of anti-Cathepsin B antibody

Four BALB/c, each weighing 30 g, were immunized with purified cathepsin B. In brief, 0.5 mg of purified recombinant cathepsin B protein in 0.5 ml PBS was thoroughly emulsified with an equal volume of Freund's complete. The emulsion was injected subcutaneously into different sites. The initial four injections, administered at 7-day intervals, employed complete adjuvant (FCA), followed by three subsequent injections with incomplete adjuvant (FICA) at the same interval. Finally, three booster doses, administered at 3-day intervals, consisted solely of the antigen. One week after the last booster dose, the animals were bled, and serum was collected. Antibody titer and specificity of action were determined using GDP and Western blot assays.

6.3. Results

Agarose gel electrophoresis of DNA

The PCR electrophoresis pattern was visualized in Fig. 6.1 using a Bio-Rad Gel documentation system, revealing a distinct DNA band at the 903 bp position.

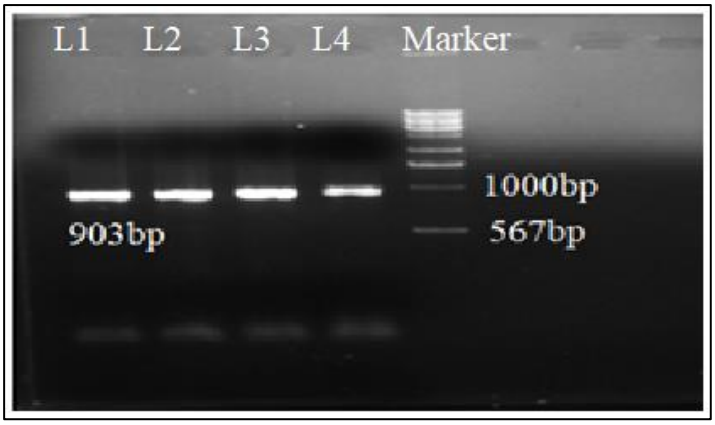


Figure 6.1: PCR amplification targeting the Cathepsin B gene resulted in positive bands observed in lanes 1 through 4.

Electrophoresis of RNA

The electrophoresis pattern of isolated RNA was visualized in Fig. 6.2 using a Bio-Rad Gel documentation system, revealing three distinct bands corresponding to 5.8S, 16S and 23S RNA of *Giardia* in the gel.

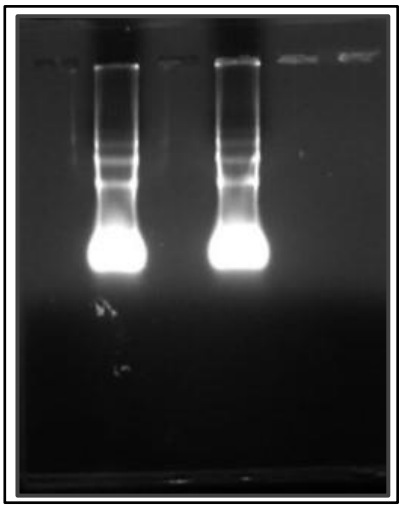


Figure 6.2: *Giardia lambia* total RNA ran in MOPS gel.

When the pET28a clones were digested with EcoRI and HindIII, the cathepsin B gene released a 903 bp band (Fig. 6.3).

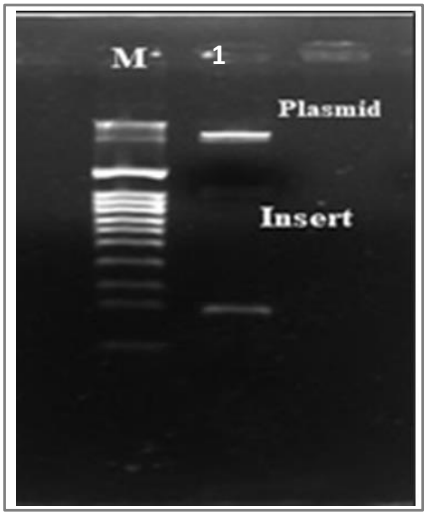


Figure 6.3: After restriction digestion, the band sizes of the plasmid and insert were observed on 1% agarose gel.

SDS-PAGE of Cathepsin B after expression

Identification and Purification of cathepsin B involved SDS-PAGE analysis, which indicated that both the supernatant and precipitate contained the target protein. However, the supernatant indicated that the recombinant proteins mainly existed in the insoluble form (Fig. 6.4). Additionally, Western blotting analysis showed that the mouse anti-His tag monoclonal antibody and HRP-labeled goat anti-mouse IgG antibody reacted with a protein of approximately 28kDa (Fig. 6.4), consistent with the theoretical value of cathepsin B. The protein detection results revealed a total protein concentration of 1.25mg/mL.

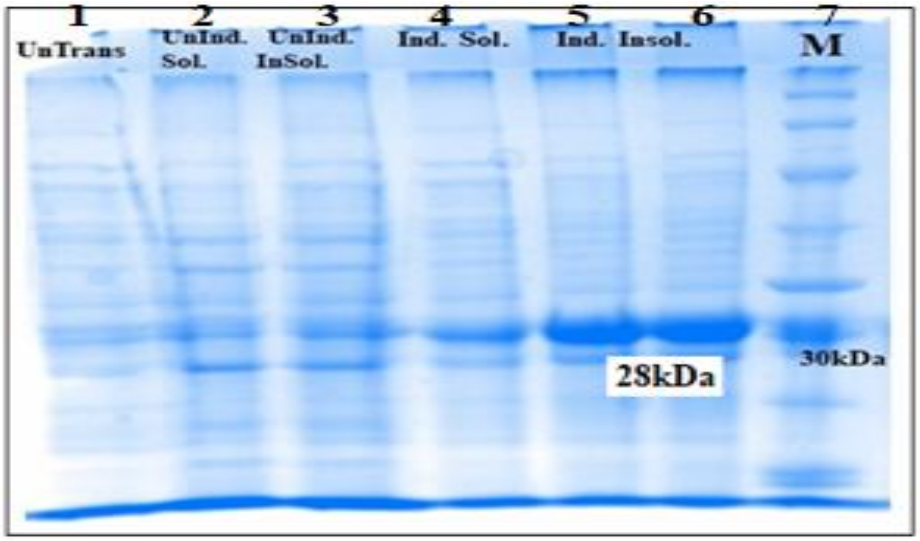


Figure 6.4: The results shows SDS-PAGE of cathepsin B gene. Lane 1 shows the untransformed sample. Lanes 2 and 3 depict uninduced soluble and insoluble fractions respectively. Lane 4 represents the induced soluble fraction. Lanes 6 and 7 display induced insoluble fractions. A standard protein marker is shown in lane 5.

Specificity of anti-Cathepsin B antibody

The specificity of the Cathepsin B antibody was evaluated. ELISA detected the antibody titer, demonstrating reactivity with recombinant Cathepsin B expressed in *E. coli* as a polyhistidine fusion protein (Fig. 6.5). Furthermore, Western blot assays with total trophozoite lysates revealed specificity to a 28kDa protein band. These results confirm that the antibody specifically targets Cathepsin B in trophozoites.

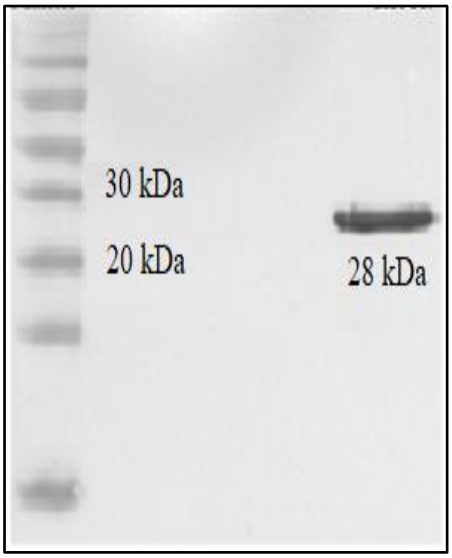


Figure 6.5: Confirmation of the specificity of anti-cathepsin B antibodies: Western blot analysis of recombinant cathepsin B proteins. The result depicts the reactivity of the anti-cathepsin B antibodies with the recombinant proteins.

6.4 Discussion

Cathepsin B-like protease activities of *Giardia* have emerged as crucial factors in host-pathogen interactions across various studies (Bhargava et al. 2015; Cotton et al. 2014). Nonetheless, the precise identification of these specific proteases remains elusive. In this study, we concentrate on the cloning and expression of cathepsin B, which belongs to cysteine proteases (CPs) family detected within the milieu of *Giardia*-host cell interactions, exploring their potential roles in disease mechanisms. Previous studies have demonstrated the up-regulation of CP14019 at both RNA and protein levels during host-parasite interactions in vitro and recent findings have revealed their release into the co-culture medium (Ringqvist et al. 2011; Ferella et al. 2014). The secretion process is bolstered by the presence of secretion signal peptides in these CPs. Moreover, their localization to vesicle-like structures scattered throughout the cytoplasm and endoplasmic reticulum (ER) suggests their involvement in protein uptake and release, as indicated by previous research (Mach et al. 1994).

The model of cathepsin B cysteine proteases' function during *Giardia*-host cell interactions suggests that these proteases, when released during host-parasite interaction, exhibit proteolytic activity capable of disrupting junctional complexes such as tight junctions (TJs) and adherens junctions (AJs). Furthermore, CPs possess the ability to traverse the epithelial barrier and degrade chemokines induced by intestinal epithelial cells, which are subsequently released on

the basolateral side in response to *Giardia* infection. Additionally, the CPs are found in their active forms, as observed during the proteolytic maturation of pro-cathepsins in previous studies (Pungercar et al. 2009). Activation can occur spontaneously or upon exposure to an acidic environment, facilitated by the pro-peptide's role as a chaperone, aiding in protein folding and regulating protease activity (Mach et al. 1994; Pungercar et al. 2009). The recombinant CPs exhibit optimal activity in an acidic pH range, typically between 5.5 and 6, consistent with the standard pH for enzymes of this class (DuBois et al. 2008; Abodeely et al. 2009; Kissoon-Singh et al. 2011). Additionally, they show a preference for the fluorescent substrate FR-AMC.

Further research into the behavior of these CPs when released collectively from *Giardia* upon contact with host cells, and the impact of their interactions with each other and with host cells on their activity, presents an intriguing subject. Maintaining the integrity of the intestinal epithelial barrier is crucial to prevent the influx of microbes and antigens into the underlying tissue (Schumann et al. 2017; Allain et al. 2017). In the context of microbial infections, maintaining the integrity of the epithelial barrier relies on the formation of a seal-like structure, achieved through the specific organization of AJC proteins. However, during infections such as those caused by *Giardia*, this arrangement is disrupted, leading to a reorganization of proteins like ZO-1, α-actinin, occludin, and F-actin. In vitro studies have shown that this reorganization correlates with an increase in the permeability of epithelial cell monolayers (Teoh et al. 2000; Chin et al. 2002). In chronic giardiasis patients, there is a noted decrease in the expression level of claudin-1, a transmembrane protein crucial for the sealing properties of tight junctions (Troeger et al. 2007). This observation underscores the impact of *Giardia* infections on the integrity of intestinal epithelia, primarily through the reorganization of proteins within the AJCs.

The ability of CPs to degrade a diverse array of substrates, including collagen I, a significant component of the extracellular matrix, suggests that *Giardia* CPs play a pivotal role in disease onset. Additionally, upon *Giardia* infection in vitro, intestinal epithelial cells (IECs) show an induction of genes encoding various chemokines. Notably, chemokines such as CCL2, CCL20, and CXCL1-3 are among those induced, with CCL20 demonstrating the highest fold change in RNA levels within the initial 90 minutes of interaction (Roxström-Lindquist et al. 2005). The distinctive chemokine profile observed targets various immune cell populations to the site of

infection. CCL20 attracts dendritic cells, T cells, and B cells, while CXCL1 to CXCL3 recruit neutrophils, and CCL2 attracts macrophages and T cells (Roxström-Lindquist et al. 2005). However, despite high transcriptional levels, the amounts of CCL20 released into the medium during interaction were low, measuring less than 50 ng/ml. This suggests post-transcriptional regulation mechanisms controlling CCL20 levels. A comparable mechanism was observed for IL-8 in a separate study, where *Giardia* CPs degraded IL-8, leading to a decrease in neutrophil chemotaxis. This effect was demonstrated in assays using a cell culture model of infection and ex vivo human biopsies (Cotton et al. 2014). Consequently, while the parasite diminishes inflammation, it simultaneously induces diarrhea and contributes to post-infectious issues such as inflammatory bowel syndrome (IBS) and food allergies. This indicates that the identified CPs play a pivotal role as virulence factors in *Giardia*. To summarize, we successfully identified and expressed the cathepsins released by *Giardia* during interaction with host intestinal epithelial cells in vitro. The recombinant CPs exhibited characteristics akin to cathepsin B, showcasing diverse kinetics and substrate preferences. The expressed CPs demonstrated the ability to degrade and reorganize junctional proteins, as well as degrade various chemokines. These findings suggest potential involvement in disrupting the intestinal epithelial barrier and modulating immune responses. Subsequent studies will delve into characterizing these proteases in *Giardia* infections in animal models and humans, aiming to pinpoint their specific roles in disease pathology.

6.5 Conclusion

In summary, this study demonstrated the cloning and expression of the Cathepsin B gene of *Giardia*. We successfully generated an antibody against the Cathepsin B gene. Further studies are needed to investigate different gene expressions at the proteomics level during *Giardia* pathogenesis.

CHAPTER-VII

ANDROGRAPHOLIDE AFFECTS *Giardia* GENES EXPRESSION BOTH TRANSCRIPTOMIC AND PROTEOMIC LEVEL

Andrographolide affects *Giardia* genes expression both transcriptomics and proteomics levels

7.1 Background

We successfully generated the antibody against the cathepsin B gene in the previous chapter. Our current focus is on investigating the effect of andrographolide on the transcriptomics of various pathogenic genes and the proteomics of Cathepsin B in *Giardia*.

Giardiasis, with approximately 280 million symptomatic cases, stands as the leading cause of human illness among parasitic diseases (Esch et al. 2013). Despite being widespread, the mechanisms and mediators of *Giardia*'s pathogenesis remain largely unclear. Human volunteer studies have provided clarity on the association between *Giardia* infection and the significance of the virulence of the infecting *Giardia* strain (Nash et al. 1987), shedding experimental light on these aspects. The molecular characterization related to strain virulence remains largely unexplored. It is evident that the majority of *Giardia* infections manifest asymptomatically. Furthermore, the infection primarily localizes to the duodenum, causing damage near the colonization sites, resulting in villus atrophy and apoptosis of surrounding cells. However, this localized damage alone cannot entirely explain the significant diarrhoea frequently linked with the disease, as it appears to affect absorption throughout a broader area of the digestive tract beyond the infection site. One of the suspected secreted mediators implicated in causing damage to the duodenum is the cathepsin B protease (Cotton et al. 2014). Cathepsin B-like proteases constitute a superfamily within the CA clan of cysteine peptidases (Turk et al. 2012). Unlike other cathepsins, cathepsin B proteases possess an additional 20 amino acid insertions termed the occluding loop. This structural feature enables them to function as either endo- or exopeptidases (Musil et al. 1991). Although 27 genes encoding cathepsin proteases have been identified in *Giardia*, the functions of the majority of these proteases remain unclear (DuBois et al. 2008). While some parasites release cathepsin B proteases to either evade or modulate their host's immune responses (Sajid et al. 2002), the specific roles of these proteases in *Giardia* are not yet fully understood.A recent study has revealed that *Giardia* trophozoites secrete cathepsin B-like proteases. These proteases contribute to the degradation of intestinal IL-8, consequently diminishing the inflammatory response initiated by the host (Cotton et al. 2014). The secreted cathepsin B protease from *Giardia* (GCATB) might additionally play a role in breaking down intestinal mucin and aiding in the attachment of trophozoites to intestinal epithelia (Rodriguez-Fuentes et al. 2006; Paget et al. 1994).

Andrographolide, a diterpenoid, is extracted from the herb *Andrographis paniculata*, with a primary presence in various parts of the plant, notably the leaves where it serves as a major bioactive phytoconstituent (Jayakumar et al. 2013). Renowned for its traditional use in Asia, andrographolide has been employed in conventional treatments for a diverse range of ailments (Chao & Lin 2010; Wong et al. 2021). Andrographolide exhibits anti-cancer properties (Banerjee et al. 2017; Sheeja & Kuttan 2007) along with anti-inflammatory and antioxidant capabilities (Mussard et al. 2019). Additionally, it has been recognized for its cardioprotective and hepatoprotective effects (Yoopan et al. 2007). Despite these known attributes of ADG, there is a notable absence of research investigating its potential as a promising antigiardial agent, specifically in relation to its ADG function. In addition to its documented properties, Andrographolide has been identified for its effectiveness against leishmanial (Sinha et al. 2000), plasmodial (Mishra et al. 2011), dengue (Edwin et al. 2016), and protozoan activities (Banerjee et al. 2017).This study aims to elucidate the impact of ADG on Giardia trophozoites, exploring its potential as an antigiardial drug. Consequently, both in vivo and ex-vivo assessments were conducted to observe the influence of ADG on parasite survival. The study also involved the evaluation of ADG effects on various genes associated with *Giardia* pathogenesis, such as oxidative stress, cell cycle, metabolic pathways, and other factors regulating genes, assessed at both the RNA and protein levels.

The study illustrates the biological impact of andrographolide as a novel antigiardial agent, emphasizing its ability to influence crucial pathogenic-regulating genes, thereby affecting the survival of parasites within the host cell.

7.2 Methodology

6.2.1 Parasite culture

Giardia lamblia trophozoites were cultured in a modified TYI-S-33 medium, which had been filter-sterilized and supplemented with 10% adult bovine serum (Diamond et al. 1978). The cultures were maintained at 37°C under microaerophilic conditions and subcultured upon reaching confluence. For the collection of parasites for experimental, the culture medium was aspirated to eliminate any unattached parasites. Subsequently, the culture tube was replenished with cold sterile medium, and trophozoites were detached by exposing them to a chilling process on ice for a duration of 15 minutes.

6.2.2 Mammalian cell line culture (INT-407)

Human intestinal epithelial (INT-407) cells were cultured in minimal essential medium containing Earle's salts (Gibco) at 37°C in a 5% CO2 atmosphere (Sousa et al. 2001). The growth medium, composed of α-Minimum Essential Medium, was supplemented with 10% fetal bovine serum (FBS) and antibiotics (10 units/ml penicillin and 10 µg/ml streptomycin). To sustain cell viability, the media were renewed every 2 days. Upon achieving confluency, cell harvesting was conducted using trypsin-EDTA (Sigma). The resulting confluent monolayers were then utilized for various applications, including electrophysiological experiments, co-culture experiments with *Giardia* parasites.

7.2.3 INT-407 and *Giardia* Co-culture

Confluent monolayers of INT-407 cells were utilized in the experiment. The cell media in the INT-407 monolayers was removed and replaced with a mixture comprising 90% complete DMEM and 10% *Giardia* medium, with or without the addition of *Giardia* trophozoites (at a concentration of 10^5 total parasites per insert). To prevent any potential contamination by parasites, control cultures were carefully maintained in a separate plate. Microscopic examination was conducted on the control inserts to confirm the absence of *Giardia* cross-contamination. Subsequently, the co-cultures were placed in an incubator at 37°C with 5% CO2 for a duration of 24 hours. Following this incubation period, the *Giardia* parasites were eliminated (Dubourg et al. 2018). In a brief procedure, the culture media was withdrawn from the inserts, and the INT-407 cell media was substituted with a blend of 99.9% complete DMEM and 0.1% *Giardia* medium, with or without the inclusion of *Giardia* supernatant.

7.2.4 RNA Extraction

RNA extraction from approximately $2x10^8$ *Giardia* trophozoites in the logarithmic growth phase involved disrupting cells with 1 ml of Trizol reagent. After rapid pelleting and a 5-minute incubation, chloroform (0.2 ml per 1 ml of Trizol) was added, followed by 15 seconds of manual agitation. The samples incubated for 5 minutes and underwent centrifugation (12,000g, 20 minutes, 2-8°C), yielding three phases. The upper aqueous phase was transferred to a new tube, and RNA precipitation was achieved by adding 0.5 ml of isopropanol, followed by a 10-minute incubation (15-30°C). Centrifugation (12,000g, 10 minutes, 2-8°C) formed a gel-like RNA pellet. A single wash with 75% ethanol, centrifugation (7,500g, 5 minutes, 2-8°C), and

air-drying purified the RNA pellet. Finally, the RNA was dissolved in 20 µl of DEPC water for downstream applications.

7.2.5 cDNA Preparation

The cDNA synthesis was performed using the Bio Bharati muLV Kit, following the manufacturer's protocols. A total reaction volume of 20 µl, including 10 ng of total RNA, was utilized for real-time PCR. The reaction mixture comprised 1 µl of RNA, 1 µl of oligo dT, 1 µl of dNTP mix, and 9 µl of water, adjusted to a total volume of 12 µl. After incubating at 65°C for 5 minutes and rapid cooling on ice, 4 µl of first-strand buffer, 1 µl of RNase inhibitor, and 2 µl of 0.1M DTT were added and incubated at room temperature. Following this, 1 µl of super RT (200 U/µl) was gently pipetted into the mixture, and the reaction was incubated at 42-52°C for 50 minutes. Finally, the inactivation was achieved by heating at 70°C for 5 minutes.

7.2.6 Real Time PCR

Numerous virulence factors play a crucial role in *Giardia* pathogenesis. Seven specific genes, namely Arginine deiminase (GL50803_112103), NADH (GL50803_9719), Kinase coding gene (GL50803_15548), FtsJ (GL50803_16993), Cathepsin (GL50803_17516), Cysteine-rich membrane protein (GL50803_113297), and Surface protein (GL50803_98861), were selected for transcriptomic analysis to confirm their differential expression using real-time PCR. These genes were chosen due to their significance in biochemical and molecular biological activities. The majority of the selected genes demonstrate upregulation upon host attachment. However, we have identified a suitable gene for normalizing mRNA levels during the *Giardia* cell cycle and other metabolic processes. The expression levels of seven genes were assessed using RT-qPCR, with the actin-related gene serving as the normalization. Primers for the seven genes were designed, and a housekeeping gene (Actin) was employed for normalization (see Table 1). The cDNA amount for RT-PCR was standardized
before real-time validation. The Taq polymerase reaction commenced by incubating at 95°C for 15 minutes to initiate the PCR process. Subsequently, 40 cycles were executed, involving denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, facilitating the amplification of DNA.

Name of the Primer	Primer sequence (5′- 3′)	Annealing temperature	Product size
ACT_F	ACATATGAGCTGCCAGATGG	53°C	110 bp
ACT_R	TCGGGGAGGCCTGCAAAC		
AD_F	GACCGTATGCACCTTGACTG	54°C	109 bp
AD_R	GCACCGACATCAATCCACTC		
NADH_F	TCCAAGTTCTCTGCGTCCAT	52°C	112 bp
NADH_R	CAACTTTAGCCATCCAGCCC		
PKC_F	GCACGCAGAGATCAACAACT	50°C	115 bp
PKC_R	ATTCTTCCAGACACCGCAGA		
FTSJ_F	TTGACACCTGAACAGCAAGC	52°C	110 bp
FTSJ_R	CTGTCGTGCCTGATCTTTGG		
CATH_F	AGCCGAGCCCCTTTTGAC	53°C	106 bp
CATH_R	GCAGGAGCAAGTGCTTGAATT		
CRP_F	AAAACAAACGAAACGCAGCC	52°C	112 bp
CRP_R	TATCCACCAAAGCCCACAGT		
SP_F	CGGAAGCAGCCAAACATGTA	54°C	110 bp
SP_R	CGCCTTCTCCCAATACAGTC		

Table 1: Primer sequences, expected PCR product sizes and annealing temperatures of the targeted gens**.**

7.2.7 *Giardia* trophozoite extracts

To obtain *Giardia* extracts, trophozoites in the logarithmic growth phase were harvested via centrifugation at 1500 rpm for 5 minutes at 4 °C. The resulting pellet underwent two rounds of washing with PBS. Following this, the cell suspension was frozen at −80 °C for 1–2 days and subsequently thawed at room temperature. Next, the trophozoites underwent sonication with five cycles of 20 seconds each. The resulting lysate was centrifuged at 12,000 g for 20 minutes. The protein concentration in the *Giardia* extracts was determined using the bicinchoninic acid assay (BCA).

7.2.8 Western blot

Western blot analysis was conducted to assess the impact of the cathepsin B gene on *Giardia* trophozoites during activation. After performing SDS-PAGE, the proteins were transferred to a nitrocellulose membrane using a voltage of 100 V for 1 hour. Subsequently, 5% skim milk was used to block the membrane in Tris-buffered Saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. The blots were then subjected to overnight incubation at 4 °C with primary antibodies targeting the proteins of interest, specifically Cathepsin B at a dilution of 1:1000. Following three washes with TBS-T, the membranes underwent a 2-hour incubation at room temperature with anti-mouse antibodies at a dilution of 1:20,000. Protein detection was accomplished using enhanced Fastcast^{TM} Acrylamide solution (BIO-RAD).

7.3 Results

7.3.1 Gene expression changes

We investigated the expression profiles of seven genes through RT-qPCR to measure their mRNA fold change levels following treatment with the active compound ADG. Our findings, illustrated in Figure 7.1, reveal distinct patterns in the expression of these genes at different doses. Notably, the protein kinase gene and surface protein exhibit an upregulation at 3 μM concentration, followed by a downregulation at 9 μM and 20 μM concentrations. Conversely, genes such as arginine deiminase, NADH, FtsJ and Cysteine-rich membrane protein exhibit a gradual decrease in expression levels across the doses of 3 μM, 9μM, and 20 μM. Interestingly, across varying concentrations of ADG treatment, the expression of the seven genes associated with pathogenic factors consistently demonstrated downregulation.

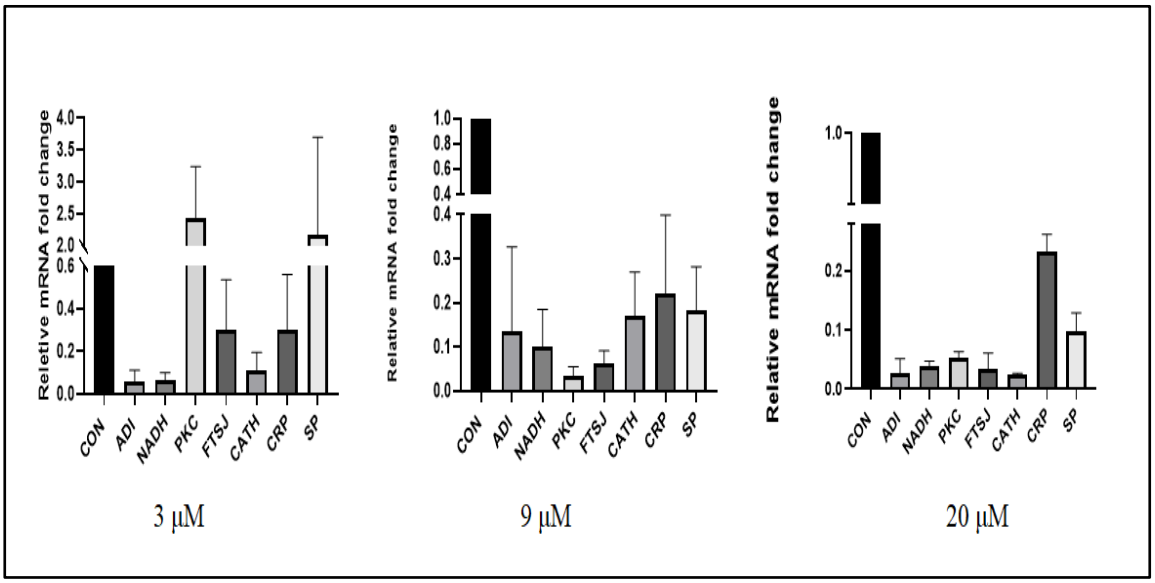


Fig. 7.1: The effect of ADG on gene expression in *Giardia* trophozoites, the mRNA fold changes using qRT-PCR at different doses: 3µM (A), 9µM (B), and 20µM (C). The genes included ADI (Arginine deiminase), NADH (NADH oxidase), PKC (Protein kinase C), FTSJ (FtsJ cell division protein), CATH (Cathepsin B), CRP (Cysteine-rich membrane protein), and SP (Surface protein).

The expression profiles of seven genes by measuring their corresponding mRNA fold change levels by RT-qPCR after the treatment of active compound ADG. We have observed the expression of those genes at different time interval. We have shown that (Fig. 7.2) the expression of arginine deiminase, NADH, and PKC gradually decreased to almost half of its normal expression at 3 hours but significantly decreased at 6 and 9 hours. Additionally, the expression of other genes, such as FtsJ, cathepsin, cysteine-rich membrane, and surface protein-regulating genes were significantly and gradually decreased at 3, 6, and 9 hours. Across various time intervals after treatment with ADG, the expression of seven pathogenic factor-regulating genes were mostly downregulated.

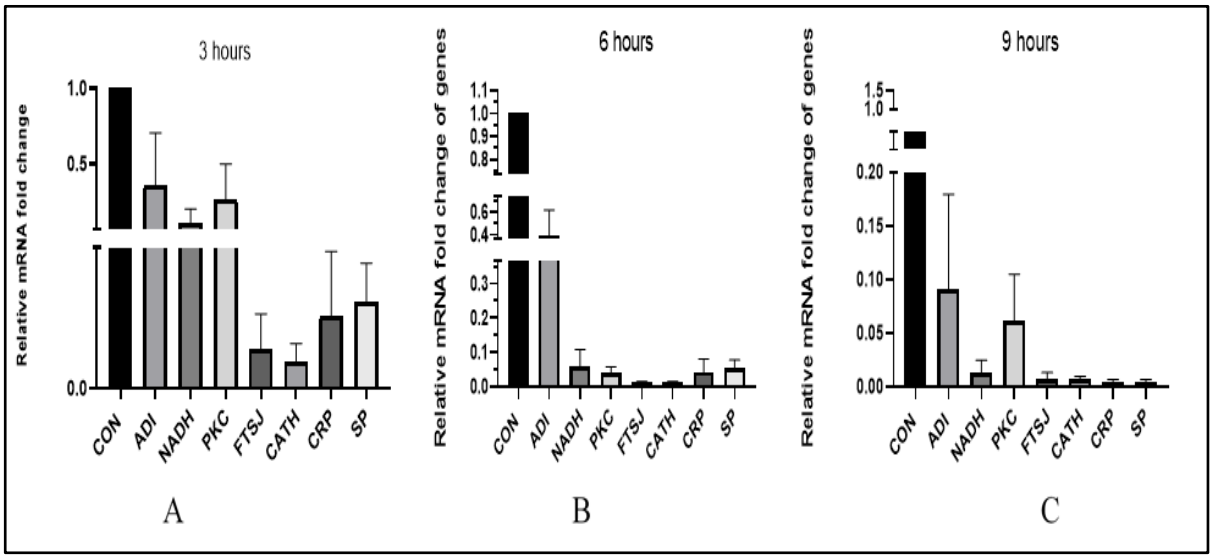


Fig.7.2: The effect of ADG on gene expression in *Giardia* trophozoites, the mRNA fold changes using qRT-PCR at different time points: 3 hours (A), 6 hours (B), and 9 hours (C).

The expression of Cathepsin B varies across different doses and time intervals. Specifically, at a 2μM concentration, there is no significant impact on protein expressio. However, as the concentration increases to 9 μM and 20 μM, the effect becomes more pronounced, resulting in a notable alteration in protein expression. Furthermore, alterations in the expression of this gene have been observed at different time points. Notably, after 24 hours of incubation, there is minimal change in the expression of the cathepsin gene. The findings suggest that at extended time intervals, the suppression of protein expression becomes more prominent.

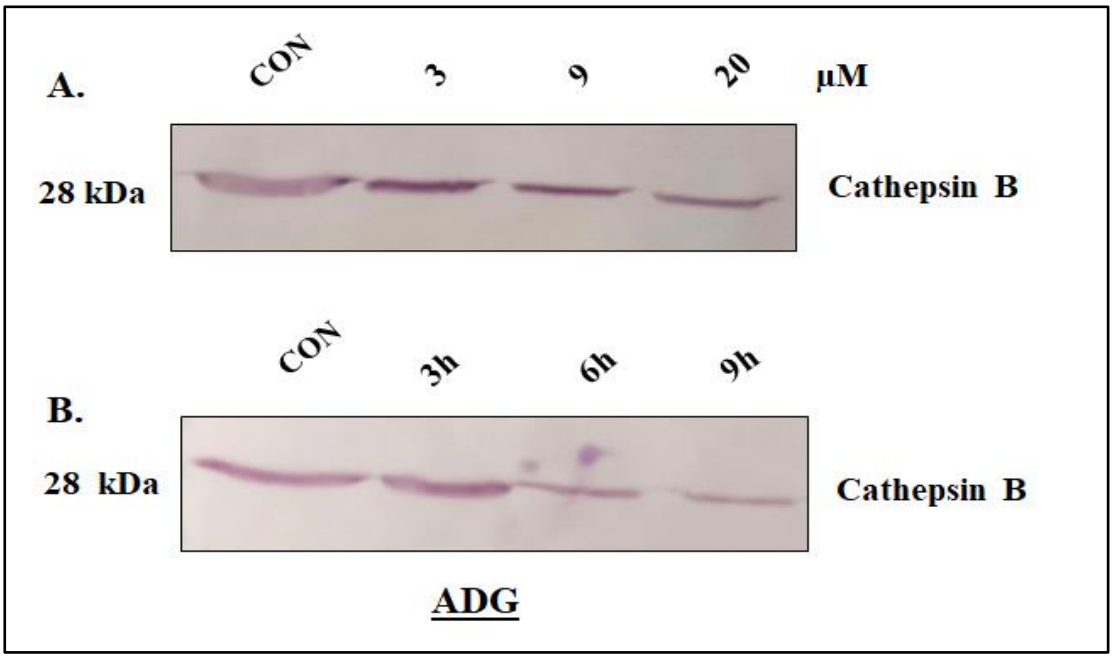


Figure 7.3: The expression patterns of the cathepsin B gene across various doses and time intervals. The gene expression at different doses (A), while the second figure represents the gene expression at distinct time intervals (B).

The study demonstrated that the drug ADG did not induce any significant changes in normal INT-407 intestinal cell culture when administered alone. This suggests that, under the tested conditions, ADG does not exert noticeable effects on normal intestinal cells. However, in a distinct scenario involving a coinfection with both intestinal cells and *Giardia* trophozoites, a notable impact was observed (Fig. 4). The *Giardia* trophozoites caused disruption to the epithelial junction between cells in this coinfection, a characteristic behavior known to compromise the integrity of the intestinal epithelium. An intriguing discovery is that treatment of the coinfection with the drug ADG at a concentration of 100µM for 24 hours resulted in the removal of all *Giardia* trophozoites, accompanied by observable changes in the cell line. This suggests that ADG possesses potent activity against *Giardia* trophozoites, leading to their elimination and subsequent alterations in the intestinal cell line.

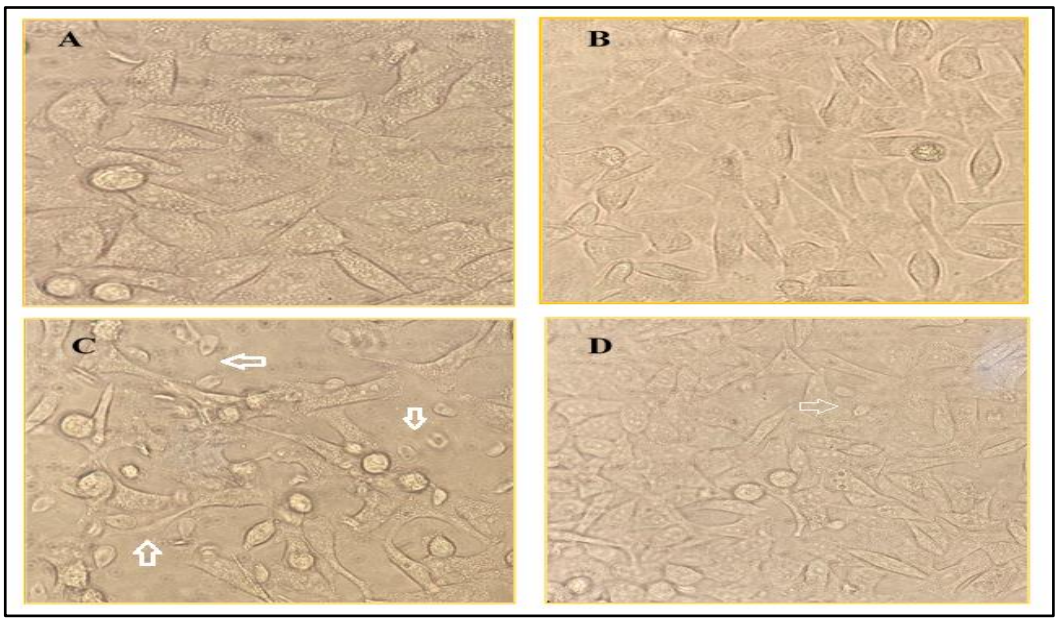


Figure 7.4: The effects of ADG on INT-407 and *Giardia* trophozoites: (A) Control INT-407 intestinal cells. (B) INT-407 cells treated with ADG. (C) Co-infection of *Giardia* with INT-407 cells, and (D) Treatment of ADG on infected cells.

7.4 Discussion

Andrographolide (ADG) has been reported to have therapeutic effects for treating various diseases (Negi et al. 2008; Woo et al. 2008). This plant contains a variety of chemical compounds, including diterpenoids, glycosides, flavonoids, lactones, and flavonoid glycosides. It has been reported that ADG possesses various biological activities, including antihepatitic, antidiarrheal, anti-inflammatory, anti-allergic, immunostimulatory, antidiabetic, antioxidant, and antimalarial properties (Hossain et al. 2014; Misra et al. 1992). Additionally, it has demonstrated hepatoprotective, anticancer, cardiovascular, antihyperglycemic (Hossain et al., 2014), and anti-HIV activities (Nanduri et al. 2004). The anti-inflammatory action of ADG involves the inhibition of nuclear transcription factor-(kappa) B, rendering it a potential therapeutic target for treating cancer and autoimmune diseases (Hidalgo et al. 2005).

In the current study, we assessed the antigiardial activity of ADG against *Giardia* trophozoites through treatment with various doses and durations. Our findings reveal that ADG substantially inhibited the expression of pathogenic factors associated with *Giardia*, inducing cell apoptosis like death and down regulating the expression of several genes. Additionally, ADG effectively inhibits the expression of pathogenic factor-regulating genes, including those involved in metabolism, cell division, surface protein regulation and cytoskeleton regulation. Further investigations revealed that ADG-mediated suppression affects these genes. To our knowledge,

this study is the first to identify ADG's ability to target various pathogenic factor-regulating genes during *Giardia* pathogenesis.

Recent studies have highlighted the significance of Cathepsin B-like protease activities from *Giardia* in host-pathogen interactions (Beatty et al. 2017; Manko et al. 2017). However, the specific proteases involved have not been identified. Our study focuses on the expression of Cathepsin-B genes, which belong to the Cysteine protease family, to identify proteomic-level expression changes following ADG treatment. Following ADG treatment, the expression level of Cathepsin-B was observed to decrease. Over different time intervals (3h, 6h and 9h), there was a gradual decrease in expression. Similarly, at various doses (3µM, 9µM and 20µM) of ADG, the gene expression level was downregulated.

The previous study provided evidence supporting the primary mechanism of *Giardia* attachment through the ventral disk, with microtubules playing a significant role. This attachment was hindered by colchicine and mebendazole, both of which affect microtubular function (Sousa et al. 2001). Our study reveals that ADG alters the morphology of trophozoites, preventing their attachment to intestinal cells. Additionally, we investigated the cytotoxic activity of ADG on intestinal cells. Following treatment with ADG in both intestinal cells and *Giardia* co-culture, we observed inhibition of trophozoite attachment to intestinal cells without any adverse effects on the intestinal cells themselves. The disease mechanisms underlying giardiasis are poorly understood, and the pathogenesis of diarrhoea and malabsorption associated with this infection is multifactorial.

7.5 Conclusion

Andrographolide inhibited the expression of various genes in *Giardia* trophozoites, particularly those involved in pathogenesis at the transcriptomics and proteomics levels. Additionally, ADG suppressed the protein expression of the Cathepsin B gene at the proteomics level. Furthermore, it inhibited the adherence of *Giardia* trophozoites in co-culture with INT-407 intestinal cells. Before using andrographolide as a drug, we need to investigate the effect and efficacy of the compound in an animal model.

CHAPTER-VIII

INVESTIGATING THE IN-VIVO IMPACT OF ANDROGRAPHOLIDE AGAINST GIARDIASIS

Investigating the in-vivo impact of Andrographolide against Giardiasis

8.1 Background

In the previous chapter, we demonstrated the effect of ADG on the expression of various pathogenic genes in *Giardia* trophozoites. The aim of this chapter is to focus on the effect and efficacy of andrographolide in animal model.

Achieving a 100% parasitological cure rate for giardiasis is uncommon, regardless of the drug or treatment regimen used, suggesting that clinical treatment failures are to be expected in some cases. Similar to bacteria, prolonged use of antimicrobials in parasites leads to the development of resistance over time. The WHO defines drug resistance as the capacity of a parasite strain to survive and multiply despite the administration and absorption of a drug at doses equal to or greater than those usually recommended, within the subject's tolerance limits (Basco & Ringwald 2000). In the context of *Giardia*, drug resistance refers to the parasite's ability to endure an antimicrobial drug dose that would typically be lethal or inhibit its growth. The term "treatment refractory" is commonly used to describe clinical infections with multiple potential causes for treatment failure, one of which could be true drug resistance in the *Giardia* isolate. Confirming drug resistance in clinical isolates is challenging due to the difficulty in establishing routine cultures (Isaac-Renton et al. 1992). Additionally, successful culturing may introduce selective bias, altering the original composition and genetic diversity of the parasite population present in the host (Andrews et al. 1992). Furthermore, resistance markers for *Giardia* are not well understood. Given these limitations, we propose that for research purposes, clinical drug-resistant giardiasis should be defined as cases where stool samples remain positive for *Giardia* more than one week after completing treatment, other causes of treatment failure have been excluded, and the risk of reinfection is very low.

Andrographis paniculata (*Ap*), a renowned herb in traditional Chinese medicine, has been extensively used to address various health issues, including viral infections, diarrhoea, dysentery, and fever. Additionally, andrographolide, derived and refined from this plant, is presently employed in China for treating inflammatory conditions such as laryngitis, upper respiratory tract infections, and rheumatoid arthritis (Xia et al. 2004; Amroyan et al. 1999; and Shen et al. 2000 & 2002). Recent research findings highlight that the anti-inflammatory

attributes of andrographolide stem from its ability to deactivate NF-κB, specifically targeting the phosphorylation of IKKβ, (Xia et al. 2004; Abu-Ghefreh et al. 2009; Bao et al. 2009). Moreover, a clinical investigation conducted by Burgos et al. 2009 observed the alleviation of symptoms associated with rheumatoid arthritis following treatment with andrographolide. Andrographolide exhibits diverse pharmacological activities encompassing anti-cancer, anti-viral, anti-thrombotic, hepatoprotective, and anti-inflammatory properties (Hossain et al. 2014). Despite its wide-ranging benefits, the utilization of ADG is somewhat constrained, partly due to its rapid absorption and metabolism in both rats and humans (Panossian et al. 2000).

Therefore, the identification of novel antigiardia agents holds significant importance for the management of giardiasis in both veterinary medicine and human (Hart et al. 2017; Tejman & Eckmann 2011). These compounds have garnered attention as possible alternatives for combined therapy with currently employed antigiardial drugs, owing to their potent activity and low toxicity to humans and other mammal hosts (Amer et al. 2014; Hassan et al. 2012).

8.2 Methodology

8.2.1. Parasite Culture

Giardia lamblia Portland1 strain (ATCCR 30888TM) was maintained in modified TYIS-33 medium supplemented with penicillin streptomycin and 10% adult bovine serum (Diamond et al. 1978; Raj et al. 2014). The medium pH was adjusted to 6.8 during the filter sterilization using 0.22µm. The culture was grown axenically under the anaerobic condition at 37°C. In preparation for the experimental inoculation, trophozoites undergoing active growth within a 48–72-hour culture were subjected to sedimentation by placing the tubes on ice for a duration of 10 minutes. Subsequently, these trophozoites were resuspended in phosphate-buffered saline (PBS-7.2) to achieve a concentration of 1×10^6 trophozoites per 0.1 ml.

8.2.2 Animals

Thirty healthy BALB/c mice, weighing 20–25 g and aged 40–60 days, were inadvertently distributed among five groups, each comprising six mice. These mice were maintained in standard animal room conditions at a temperature of 25 ± 2˚C, with constant access to food and water.

8.2.3 Induction of Animal Model Giardiasis

To induce the animal model of giardiasis, each tested group of mice received 200 µL of *G. lamblia* trophozoites (2×10^4/PBS) orally through flexible plastic gavage needles. To validate the infection, mice were observed daily under a microscope starting from the initial day of inoculation until the identification of *Giardia* cysts in their feces. The detection of cysts in the fecal samples confirmed the successful infection of the mice with the parasite.

8.2.4 Experimental Design and Follow-up of the Animals

The animals were initially classified into five groups, each comprising six animals. Group I (Control): the negative control and received treatment with normal saline/DMSO. In Group II (*Giardia* infected), mice were orally challenged with a single dose of *Giardia* trophozoites through orogastric gavage. Groups III and IV (Drug treated) included infected mice treated with a single lower dose of ADG (10 mg/kg) and a higher dose of ADG (20 mg/kg), respectively, administered for a duration of 5 days. Group V, the positive control (MTZ), received treatment with metronidazole (MTZ). Animals in Groups II, III, IV, and V received an oral administration of a single dose of their respective *Giardia* strains for a period of 7 days. Following the designated treatments in all groups, the count of *Giardia* trophozoites was monitored.

8.3 Results

In this study, we assessed cyst shedding data from five groups. Through ongoing microscopic examination of stool samples, we observed a noteworthy decrease in the number of cysts in mice treated with the ADG extract, as illustrated in Figure 8.1. Particularly, at a dosage of 20 mg/kg body weight, a significant reduction in cysts was observed.

Immunocytochemistry analysis of *Giardia* revealed trophozoites exhibiting a deep brown coloration. In the control group mice (Fig. 8.1A), no trophozoites were detected. However, in mice infected with *Giardia* trophozoites (Fig. 8.1B), trophozoites were exclusively observed on the brush border. Following a five-day treatment with ADG in infected mice, there was a complete removal of trophozoites from intestinal tissue (Fig. 8.1C). Similarly, mice treated with MTZ also displayed a full recovery from trophozoite presence (Fig. 8.1D). Furthermore, the administration of ADG at a dosage of 20 mg/kg of body weight to mice infected with *Giardia* resulted in a lethal effect after 5 days. Intriguingly, when ADG was administered at the same dose to mice infected with *Giardia*, the parasites were eliminated after five days. In the in vivo

study of ADG in *Giardia* at a dosage of 20 mg/kg of body weight, the compound demonstrated efficacy in eliminating the parasites within a five-day period.

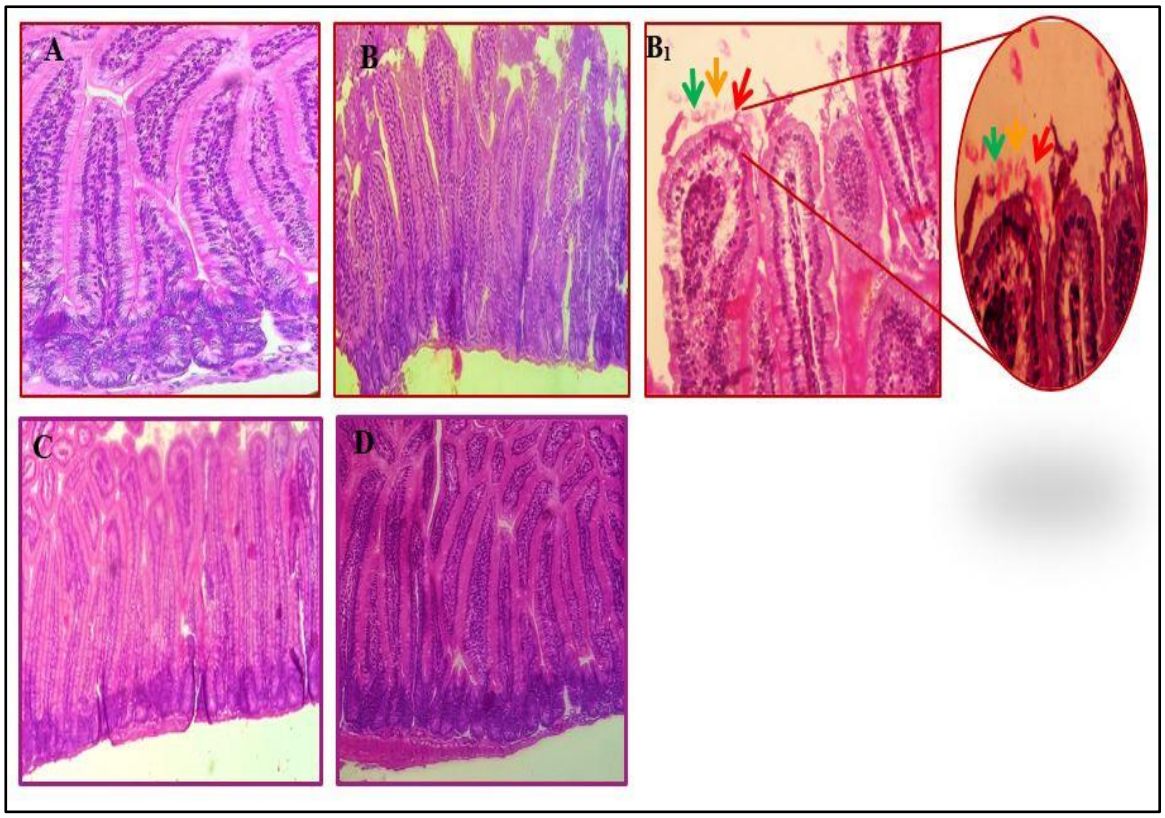


Figure 8.1: Histological sections of the small intestine from examined mice under a light microscope reveal the following: A. Healthy, non-infected tissue. B. Tissue from mice infected with *Giardia* trophozoites, untreated, displaying apparent villus atrophy. Arrows indicate the presence of *Giardia* trophozoites. C. Tissue from mice infected with *Giardia* trophozoites, treated with ADG, where no *Giardia* trophozoites are observable. D. Tissue from mice infected with *Giardia* trophozoites, treated with Metronidazole, showing an absence of *Giardia* trophozoites.

8.4 Discussion

A paniculata (Burm. f.) Nees, a medicinal plant, has a well-established traditional reputation for its diverse therapeutic properties, including antidiabetic, anti-inflammatory, hepatoprotective, antispasmodic, and antioxidant effects (Koteswara Rao et al. 2004; Niranjan et al. 2010). This plant is notably rich in key constituents such as diterpenoids, flavonoids, and polyphenols (Koteswara Rao et al. 2004). One particularly noteworthy compound found abundantly in A. paniculata is ADG. ADG has been utilized for an extended period in addressing a range of diseases, encompassing both bacterial and viral infections. In a study conducted by Arifullah et al. 2013, the antibacterial properties of ADG were demonstrated. Previous research has not reported substantial toxicity associated with ADG, as indicated by clinical histopathological observations.

The mechanisms driving the antigiardial effects of andrographolide in the in vivo mice model are likely intricate and diverse. Earlier research has proposed several potential avenues through which andrographolide exerts its influence, including disrupting the integrity of the parasite's cell membrane, impeding its attachment to host cells, and modulating vital metabolic pathways crucial for its survival. Furthermore, the compound's immunomodulatory and anti-inflammatory properties could play a role in the notable reduction of trophozoite counts by bolstering the host's defense mechanisms. These combined actions suggest a comprehensive and multifaceted approach by which andrographolide combats giardial infection in the experimental mice model.The noteworthy decrease in *Giardia* trophozoites documented in this study carries optimistic implications for advancing andrographolide as a prospective therapeutic solution for giardiasis. Additional research is essential to unveil the exact molecular mechanisms accountable for the antigiardial effects of andrographolide. Investigations into optimizing parameters, such as the ideal dosage, treatment duration, and potential synergies when co-administered with existing antigiardial medications, should be pursued. Moreover, a comprehensive assessment of safety and toxicity profiles is imperative before contemplating the clinical application of andrographolide.

The application of andrographolide at a dosage of 20mg/kg body weight showed a notable decrease in *Giardia* trophozoites within an in vivo mice model. This encouraging outcome sets the stage for additional exploration and advancement of andrographolide as a prospective therapeutic approach for giardiasis. As our investigation into andrographolide's anti-parasitic mechanisms progresses, the persuasive potential to incorporate this natural compound into the array against giardiasis becomes increasingly evident.

8.5 Conclusion

The objective of this study was to evaluate the potential antigiardial activity of ADG in vivo, exploring its viability as an alternative remedy to existing commercial treatments. Our findings provide significant insights, revealing that ADG exhibits giardicidal effects. However, as the initial report on the in vivo impact of ADG on *G. lamblia*, further studies, preferably with an extended treatment duration, are necessary to deepen our understanding of its efficacy and potential as a substitute for current treatments.

CHAPTER-IX

SUMMARY

9.1. Summary

Giardia parasites are of significant importance due to their widespread prevalence and the health issues they cause. These parasites, particularly *Giardia* lamblia, are responsible for causing giardiasis, a common intestinal infection affecting millions of people globally. giardiasis can lead to symptoms such as diarrhea, abdominal cramps, nausea, and dehydration, posing a substantial burden on public health systems, especially in developing countries. Additionally, giardiasis can be particularly severe in vulnerable populations such as young children, the elderly, and individuals with compromised immune systems. Understanding the biology, transmission, and treatment of *Giardia* parasites is crucial for effective disease management, prevention strategies, and improving public health outcomes.

Traditional plants hold significant importance due to their long history of use in various cultures for medicinal, culinary, and cultural purposes. These plants often contain bioactive compounds with potential therapeutic properties, making them valuable resources for drug discovery and development. Studying traditional plants can provide insights into indigenous knowledge systems, biodiversity conservation, and sustainable healthcare practices. Additionally, traditional plants offer opportunities for exploring alternative and complementary medicine approaches, which can be particularly relevant in regions with limited access to modern healthcare services. Understanding the importance of traditional plants can help bridge the gap between traditional and modern medicine, fostering collaboration and innovation in healthcare research and practice.

Drug resistance in parasites is a significant problem that undermines the effectiveness of treatments for various infectious diseases. Parasites, including protozoa, helminths, and other organisms, can develop resistance to commonly used antiparasitic drugs through various mechanisms. This resistance can arise due to genetic mutations, decreased drug uptake, increased drug efflux, or alterations in drug targets. As a result, infections become more difficult to treat, leading to treatment failure, prolonged illness, increased healthcare costs, and potentially higher mortality rates. Drug resistance in parasites poses a considerable challenge to global health efforts, particularly in regions where these infections are endemic and resources for surveillance, diagnosis, and treatment are limited. Addressing the problem of drug resistance requires a multifaceted approach that includes surveillance, research into alternative treatments, improved drug stewardship, and efforts to prevent the spread of resistant parasites.

Alternative drugs play a crucial role in addressing various health challenges, particularly in the context of drug resistance and adverse side effects associated with conventional medications.

These alternative drugs, which can include natural products, herbal remedies, traditional medicines, and novel compounds derived from various sources, offer new therapeutic options for treating diseases and improving patient outcomes. They may provide benefits such as reduced toxicity, enhanced efficacy, broader spectrum of activity, and lower risk of resistance development compared to conventional drugs. Additionally, alternative drugs often have cultural significance and are valued for their holistic approach to health and well-being. Furthermore, alternative drugs offer opportunities for drug discovery and innovation, driving research into new treatment modalities and contributing to the development of more sustainable and accessible healthcare solutions. Overall, alternative drugs play a vital role in complementing traditional medical practices and expanding the range of treatment options available to patients.

The kalmegh plant, scientifically known as *Andrographis paniculata*, has garnered attention for its diverse medicinal properties across various traditional medicine systems. Renowned for centuries, its medicinal activity spans a broad spectrum, encompassing anti-inflammatory, antiviral, antibacterial, antioxidant, immunomodulatory, antimalarial, hepatoprotective, and potential anticancer effects. With its anti-inflammatory prowess, Andrographis holds promise in mitigating conditions like arthritis, asthma, and inflammatory bowel disease. Its antiviral properties extend to combatting influenza, herpes simplex, and HIV infections. Furthermore, its antibacterial activity contributes to tackling antibiotic-resistant strains. Acting as an antioxidant, Andrographis scavenges free radicals, potentially shielding against chronic diseases such as cancer and cardiovascular ailments. Moreover, its immunomodulatory function bolsters the body's immune defenses against infections. Andrographis extracts have also shown efficacy against malaria parasites, underscoring its potential in antimalarial strategies. Additionally, it serves as a hepatoprotective agent, safeguarding the liver from diverse insults. Preclinical studies hint at its anticancer potential, marking it as a candidate for cancer therapy. In essence, Andrographis's multifaceted medicinal activity underscores its significance in traditional medicine and its growing interest in modern scientific exploration for therapeutic applications.

During our study, we have demonstrated the potent antigiardial effect of the *Andrographis* plant, unveiling its multifaceted impact on *Giardia* parasites. Our findings revealed that the extracts induce DNA damage, disrupt the cell cycle, provoke morphology alterations, and stimulate the generation of ROS) in *Giardia* trophozoites. These collective actions underscore the broad spectrum of anti-parasitic mechanisms exerted by this plant, highlighting its potential as a therapeutic agent against *Giardia* infections. The observed effects offer valuable insights

into the intricate interactions between Andrographis bioactive components and *Giardia* parasites, paving the way for further exploration of Andrographis-based interventions for combating giardiasis.

In our study, we have demonstrated the remarkable antigiardia activity of andrographolide, an active compound derived from the *Andrographis* plant, both in vitro and in vivo. Our findings unveil its potent effects, which include inducing DNA damage, arresting the cell cycle, eliciting morphology changes, and inhibiting the generation of reactive oxygen species (ROS). Furthermore, andrographolide exhibits the capability to suppress gene expression associated with pathogenesis in *Giardia* parasites. These comprehensive actions underscore the multifaceted nature of andrographolide's antigiardia properties, suggesting its promising potential as a therapeutic intervention against giardiasis. The observed effects shed light on the intricate mechanisms underlying the interaction between andrographolide and *Giardia* parasites, opening avenues for further exploration of its clinical applications in combating this parasitic infection. Our study highlights the significant potential of *Andrographis* plant and its active compound, andrographolide, as a promising alternative treatment for giardiasis. Through rigorous in vitro and in vivo investigations, we have demonstrated the potent antigiardia activity of andrographolide, shedding light on its multifaceted mechanisms of action including DNA damage induction, cell cycle arrest, morphological alterations, and inhibition of reactive oxygen species generation. These findings not only underscore the therapeutic efficacy of andrographolide against *Giardia* parasites but also provide new hope in the quest for novel treatments for giardiasis. By offering a natural and effective alternative to conventional drugs, *Andrographis* plant-derived compounds hold promise in addressing the challenges of drug resistance and adverse side effects associated with existing therapies. As such, our study paves the way for further research and clinical trials to harness the full therapeutic potential of andrographolide, offering new avenues for the management and control of giardiasis, a prevalent and burdensome parasitic infection worldwide.

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PUBLICATIONS

PUBLICATIONS AND CONFERENCES

1. **Haldar T**, Sardar SK, Ghosal A, Prasad A, Saito-Nakano Y, Dutta S, Nozaki T, Ganguly S (2024). Andrographolide induced cytotoxicity and cell cycle arrest in *Giardia* trophozoites. Exp Parasitol, 122: 2567-84. https://doi.org/10.1016/j.exppara.2024.108773

2. **Haldar T**, Sardar SK, Ghosal A, Das K, Saito-Nakano Y, Dutta S, Nozaki T, Ganguly S (2024). Unveiling the anti-giardial properties of *Andrographis paniculata* leaf extract through *in vitro* Studies. Tropical Biomedicine (Accepted).

3. Sardar SK, Ghosal A, **Haldar T**, Prasad A, Mal S, Saito-Nakano Y, Kobayashi S, Dutta S, Nozaki T, Ganguly S (2024).Genetic characterization of the *Entamoeba moshkovskii* population based on different potential genetic markers. Parasitology, 151(4), 429–439. https://doi.org/10.1017/S003118202400026X

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CONFERENCE: POSTER PRESENTATION

1. **Haldar T,** Ganguly S. Unveiling cytotoxic activity and therapeutic potential of andrographolide against Giardiasis. Green Bio-Merge. Brainware University, February 22-24, 2024, India.

2. **Haldar T,** Ganguly S. Cytotoxic activity of andrographolide, an active compound of *Andrographis paniculata* against *Giardia lamblia.* 16th Asian Conference on Diarrhoeal Diseases and Nutrition (ASCODD). NICED Kolkata, November 11-13, 2022 India.

3. **Haldar T**, Maruf M, Ganguly S. Andrographolide, an active compound of *Andrographis paniculata* shows antigiardial activity *in vitro*. National Conference of Indian Academy of Tropical Parasitology (TROPCON) September 21-24, 2022, India.

4. Actively participated as a volunteer in the India International Science Festival (IISF) Conference 2019, Kolkata, India

5. Actively participated as a volunteer in the Antimicrobial Resistance (AMR) conference in 2019, conducted by ICMR-NICED, Kolkata, India.

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Andrographolide induced cytotoxicity and cell cycle arrest in *Giardia* trophozoites

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ABSTRACT

Giardiasis is a prevalent parasitic diarrheal disease caused by *Giardia lamblia*, affecting people worldwide. Recently, the availability of several drugs for its treatment has highlighted issues such as multidrug resistance, limited effectiveness and undesirable side effects. Therefore, it is necessary to develop alternative new drugs and treatment strategies that can enhance therapeutic outcomes and effectively treat giardiasis. Natural compounds show promise in the search for more potent anti-giardial agents. Our investigation focused on the effect of Andrographolide (ADG), an active compound of the *Andrographis paniculata* plant, on *Giardia lamblia*, assessing trophozoite growth, morphological changes, cell cycle arrest, DNA damage and inhibition of gene expression associated with pathogenic factors. ADG demonstrated anti-*Giardia* activity almost equivalent to the reference drug metronidazole, with an IC_{50} value of 4.99 μM after 24 h of incubation. In cytotoxicity assessments and morphological examinations, it showed significant alterations in trophozoite shape and size and effectively hindered the adhesion of trophozoites. It also caused excessive ROS generation, DNA damage, cell cycle arrest and inhibited the gene expression related to pathogenesis. Our findings have revealed the anti-giardial efficacy of ADG, suggesting its potential as an agent against *Giardia* infections. This could offer a natural and low-risk treatment option for giardiasis, reducing the risk of side effects and drug resistance.

1. Introduction

Giardia lamblia is one of the most frequent protozoan parasites that cause giardiasis worldwide. According to WHO reports annually 280 million people have been infected globally (Carter et al., 2018). Giardiasis is becoming an increasingly prevalent issue, particularly in developing country, as it continues to emerge and spread within human populations. In developed countries, the prevalence rate of giardiasis is between 2 and 5%, while in developing countries ranges from 20 to 30% (Lalle and Hanevik, 2018). The disease is typically transmitted indirectly through the consumption of food or water that has been contaminated with cysts or through person-to-person contact, especially among individuals living in unhygienic conditions. Although giardiasis can affect individuals of all ages and display symptoms, it tends to affect children more frequently than adults (Belkessa et al., 2021). Giardiasis can either be asymptomatic or result in symptoms such as diarrhoea, vomiting, flatulence, anorexia, and crampy abdominal pain during its acute phase (Escobedo et al., 2018). In addition to gastrointestinal symptoms, it may also lead to extra-intestinal manifestations such as fever, maculopapular rash, pulmonary infiltrate, lymphadenopathy, polyarthritis, urticaria and growth retardation. Metronidazole is considered the first-line treatment for giardiasis, but alternative medications like albendazole, furazolidone and tinidazole are also used (Beer et al., 2017). Adverse effects of common drugs results headaches, dizziness, metallic taste, low efficacy and treatment failure have been reported as potential issues that cannot be overlooked (Starrs and Yenigun, 2021). The development of resistance to chemotherapeutic drugs among parasites underscores the critical importance of exploring alternative therapeutic approaches. One such approach involves investigating natural plant products as potential novel anti-parasitic agents (Anthony et al., 2005).

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Andrographis paniculata (Burm. F.) Nees, a member of the Acanthaceae family, is a plant that is commonly found in several Asian countries including China, India, Thailand, and Sri Lanka (Hossain et al., 2014). It has a long history of medicinal use in both Indian and Oriental medicine. Ancient Ayurvedic texts have highlighted its potential as an herb for addressing neoplasms and have documented at least 26 Ayurvedic formulations that incorporate it in the treatment of liver disorders (Okhuarobo et al., 2014). The plant, commonly referred to as the "king of bitters," is widely recognized for its highly bitter characteristics and has been traditionally employed as a treatment for various ailments, including the common fever, cold, dysentery, tonsillitis, liver ailments, diarrhoea, inflammation, herpes, and many others (Hossain et al., 2014). The plant contains various active compounds, such as diterpene lactones, flavonoids, and polyphenols, as reported in studies (Okhuarobo et al., 2014). Andrographolide (ADG), a diterpenoid lactone with the chemical formula $C_{20}H_{30}O_5$, serves as the primary compound responsible for the plant's therapeutic properties (Chao and Lin, 2010). It is primarily concentrated in the leaves of the plant and can be readily isolated from plant crude extracts as a crystalline solid (Varma et al., 2011). ADG demonstrates an extremely broad spectrum of biological activities. Recent reports suggest that it possesses anti-tumour, cardio-protective, anti-HIV, antioxidant, anti-inflammatory, immunomodulatory, antibacterial, cytotoxic, neuroprotective, and hepatoprotective properties (Li et al., 2022). Furthermore, it has been shown to exhibit antimicrobial activity against bacteria and viruses.

This study underscores the effectiveness of andrographolide against giardiasis. Our research demonstrates its ability to induce cytotoxic effects, morphological changes, ROS production, DNA damage and cell cycle arrest in trophozoites. These results highlight potent anti-giardial properties of andrographolide, suggesting its potential for novel drug therapies for giardiasis.

2. Materials and methods

2.1. Parasite culture

In all experiments, *G. lamblia* trophozoites (ATCC 30888, Portland 1 strain) were used. *Giardia* trophozoites axenic culture were maintained in TYIS-33 medium at 37 °C. The media was supplement with penicillin (100 U/ml) streptomycin (100 mg/ml) antibiotic and enriched with adult bovine serum 10% (Raj et al., 2014). The subculture was maintained every 48 h and only the trophozoites from the logarithmic phase were used for all experiment.

2.2. Intestinal cell culture

Intestinal cells 407 (INT-407), originating from human embryonic tissue of the jejunum and ileum, were cultured in 25 cm^2 flasks at 37 °C. DMEM (Dulbecco's modified Eagle's media) was used for culturing the cells, and it was enriched with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin.

2.3. Andrographolide

The active compound Andrographolide is derived from the *Andrographis paniculata* plant. We have purchased this compound from Sigma Aldrich (USA purity ≥98% 365645).

2.4. In vitro cytotoxicity assay

We used trypan blue to assess the cytotoxic effect of ADG on *Giardia* trophozoites. ADG was diluted to a concentration lower than 0.1% with DMSO in the final solution. In this assay, following treatment with different concentrations of ADG (3–20 μM), tubes were cooled for 10 min and then centrifuged at 250×*g* for 10 min at 4 °C. The parasites were washed with 1X PBS (pH 7.2) and subsequently incubated in a 0.4% trypan blue solution at room temperature for 5 min. Finally, the parasites were counted under a microscope using a hemocytometer (Carvalho et al., 2014). We conducted the experiment by exposing the parasites to various doses and times of ADG and assessing the mortality. After 24 h of treatment, the IC_{50} value for trophozoites was determined by using nonlinear regression online ICEstimator software (http://www.antimalarial-icestimator.net/runregression1.2.htm).

2.5. Scanning Electron Microscopy (SEM)

Scanning electron microscopy is employed to observe and analyse morphological alterations following the administration of ADG. The ADG treated trophozoites cells were washed with 1X PBS (pH 7.2) buffer by centrifugation at 1500 rpm for 10 min. The cells were then fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4 °C. After fixation, the samples were dehydrated through a serial dilution using alcohol solutions with increasing concentrations, ranging from 30%, 50%, 70%, 90% and 100% ethanol. To remove any remaining alcohol from the sample without causing structural damage, 2 ml of hexamethyl disilazane were added and incubated for 10 min. After adding 1 ml hexamethyl disilazane the samples were incubated for an additional hour. Finally, the samples were kept in a fume hood overnight for proper safety. To enable SEM imaging, the sample was mounted onto a stub using conductive adhesive and coated with a thin layer of gold using evaporation techniques. This coating process enhances the conductivity of the sample, which is crucial for SEM imaging. The prepared samples were imaged using SEM.

2.6. The nuclei staining

DAPI (4′, 6-diamidino-2-phenylindole) staining was employed to investigate DNA damage in trophozoites following treatment with ADG. The trophozoite culture treated with ADG was harvested and then subjected to centrifugation at a lower speed (1500 rpm) for 10 min to pellet the parasites. Gently removed the supernatant and pellets were washed by resuspending with 1X PBS (pH 7.2). Following fixation with 4% formaldehyde, the cells were incubated with a blocking buffer that included 0.1% Triton X-100 for a duration of 1 h. DAPI stock solution was added directly to the resuspended pellet at a final concentration of 1–10 μg/ml and gently mixed to ensure uniform distribution. The samples were incubated in dark for 30 min at room temperature to allow DAPI to penetrate the *Giardia* trophozoites nuclei. After the incubation period, the sample was centrifuged at a low speed (1500 rpm) for 10 min to pellet the stained parasites. The pellet was washed by resuspending with 1X PBS (pH 7.2) and the centrifugation step was repeated at least three times to remove excess DAPI or any non-specifically bound dye. The coverslip-mounted samples were prepared on glass slides and the slides were then imaged using a Confocal Microscope (Zeiss LSM 710).

2.7. Cell cycle study

To explore the impact of ADG on *Giardia* cell cycle, we utilized a PI/RNase solution (Invitrogen) in flow cytometry analysis to detect potential cell cycle arrest. *Giardia* trophozoite cells were treated with ADG, harvested and then washing with 1X PBS (pH 7.2) through centrifugation at 1000×*g* for 10 min at 4 °C. The resulting pellet was dissolved in 70% ethanol and fixed overnight at 4 °C. During fixation, 100 μl of 70% ethanol was added repeatedly while vortexing to prevent cell clumping. After centrifugation 1500 rpm for 10 min, 400 μl of PI/RNase staining solution was added and incubated for 1 h at 37 °C. Finally, the cell cycle was analyzed by flow cytometer (BD FACSAria™ II).

2.8. Determination of intracellular ROS generation

We performed a DCFDA assay to observe ROS (Reactive Oxygen Species) generation in *Giardia* trophozoites after treatment with ADG.

For each set, 4×10^6 *Giardia* trophozoites were collected and suspended in 1X PBS (pH 7.2). Following this, 2 μl of DCFDA (2′-7′-Dichlorodihydrofluorescein diacetate) (Invitrogen) from 100 μM stock solution was added to each case. Subsequently, all the sets were incubated at 37 °C for 15 min in absence of light. After the incubation, the samples underwent three washes with 1X PBS (pH 7.2), and finally, slides were prepared for confocal examination. We used a Confocal Microscope (Zeiss LSM 710) for data generation with an emission wavelength of 530 nm and an excitation wavelength of 488 nm.

2.9. RNA isolation and cDNA preparation

We performed real-time PCR to investigate the differential expression of various genes following ADG treatment. Total ADG treated and non-treated RNA was extracted from approximately 5×10^7 trophozoites using a TRIZOL kit (Invitrogen) according to its manufacture protocols. The optical density at 260 nm and 280 nm was measured to determine the concentration and purity of the RNA. The cDNA was synthesized by using kit following it manufacture protocols (Bio Bharati muLV Kit). Total 20-μl reaction volume, containing 10 ng of total RNA, was used for real-time PCR. The reaction mixture consisted of 2 μl RNA, 1 μl of oligo dT, 1 μl dNTP mix, and 8 μl water, adjusted to a total volume of 20 μl. After incubating at 65 °C for 5 min and rapid cooling on ice, 5 μl first strand buffer, 1 μl RNase inhibitor, and 2 μl 0.1M DTT were added and incubated at room temperature. Following this, 1 μl super RT (200 U/μl) was gently pipetted into the mixture, which was then incubated at 42–52 °C for 50 min. The reaction was subsequently inactivated by heating it at 70 °C for 5 min after completion.

2.10. Real time PCR

Numerous virulent factors predominantly help *Giardia* in pathogenesis. Seven specific important virulent genes like Arginine deiminase (GL50803_112103), NADH (GL50803_9719), Kinase coding gene (GL50803_15548), FtsJ (GL50803_16993), Cathepsin (GL50803_17516), Cysteine-rich membrane protein (GL50803_113297) and Surface protein (GL50803_98861) were chosen for transcriptomic analysis to validate their differential expression using real-time PCR, given their importance in biochemical and molecular biological activities. The majority of selected genes exhibit upregulation upon host attachment. However, we have selected an appropriate gene for normalization of the mRNA levels during the *Giardia* cell cycle and other metabolic process. The expression levels of seven genes were measured by RT-qPCR using the actin-related gene as the normalize. The primers for seven genes have been designed and a housekeeping gene (Actin) was used for normalization (Table 1). The amount of cDNA for RT-PCR was standardized before the real-time validation. The Taq polymerase reaction was initiated by incubating at 95 °C for 15 min to initiate the PCR process. Subsequently, 40 cycles were performed, involving denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, to amplify the DNA.

Table 1
The primer sequences of seven genes along with actin were used in real-time PCR.

Genes	Primer Sequences
ACT: F	**ACATATGAGCTGCCAGATGG**
ACT: R	**TCGGGGAGGCCTGCAAAC**
AD: F	**GACCGTATGCACCTTGACTG**
AD: R	**GCACCGACATCAATCCACTC**
NADH: F	**TCCAAGTTCTCTGCGTCCAT**
NADH: R	**CAACTTTAGCCATCCAGCCC**
PKC: F	**GCACGCAGAGATCAACAACT**
PKC: R	**ATTCTTCCAGACACCGCAGA**
FTSJ: F	**TTGACACCTGAACAGCAAGC**
FTSJ: R	**CTGTCGTGCCTGATCTTTGG**
CATH: F	**AGCCGAGCCCCTTTTGAC**
CATH: R	**GCAGGAGCAAGTGCTTGAATT**
HCM: F	**AAAACAAACGAAACGCAGCC**
HCM: R	**TATCCACCAAAGCCCACAGT**
SP: F	**CGGAAGCAGCCAAACATGTA**
SP: R	**CGCCTTCTCCCAATACAGTC**

3. Results

3.1. Cytotoxic effect

ADG demonstrated a potent, dose-dependent inhibition of parasites compared to the control. After 24 h, the IC_{50} value for ADG is 4.99 μM, which is almost similar to metronidazole (Argüello-García et al., 2018). Additionally, it was observed that the 80 μM ADG concentration did not show any cytotoxic effect on INT-407, normal human intestinal cells. The results showed (Fig. 1) a significant reduction in trophozoite as the concentration of ADG increased, indicating a dose and time-dependent effect. With an ADG concentration of 3 μM, the average percentage of cell death reached 36.35%. Furthermore, the study revealed that increasing the ADG concentration to 6 μM resulted death rate of 44.1%, while a concentration of 9 μM cell death rate of 62.76% and 15 μM contains 76.47% of cell death. Notably, 91.18% of the cells death occur even at the highest tested concentration of ADG 20 μM for 24 h.

The study examined the mortality rate of *Giardia* trophozoites when exposed to ADG IC_{50} concentration the trophozoites were monitored at different time intervals to assess the impact of the treatment. Initially, after 4 h of treatment, no significant effect on trophozoites was observed. However, in Fig. 2 after 12 h of treatment, the activity of the compound noticeably increased, resulting in a higher frequency of trophozoite death over time. As the incubation period was prolonged to 48 h and 72 h, the cell death reached its maximum. Particularly, at the 72-h time point, the ADG exhibited its highest level of activity, causing 84.0% cell death.

3.2. Morphology changes

We observed that the administration of ADG could induce changes in the shape and size of *Giardia* trophozoites. Particularly, cells treated

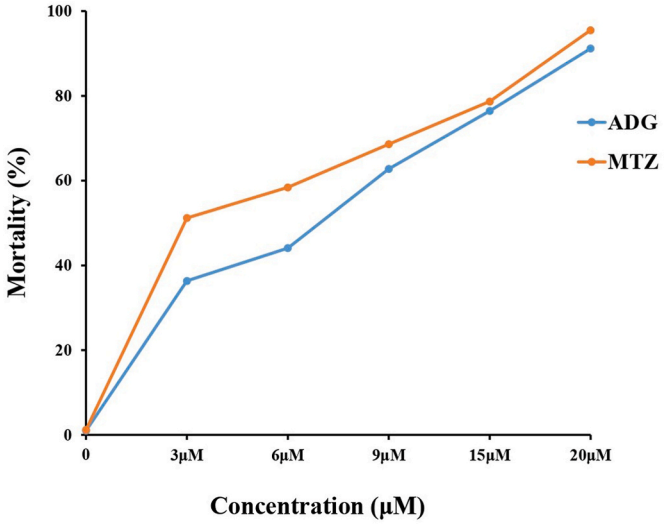

Fig. 1. The assessment of cell death activity of ADG compared to metronidazole. *Giardia lamblia* trophozoites were treated with ADG and metronidazole at various concentrations (3–20 μM) 0.1% DMSO control for 24 h of incubation. The dose dependent inhibitory effects of ADG were evaluated using trypan blue assays. The experiment was conducted in triplicate and the IC_{50} growth inhibition of ADG against trophozoites was 4.99 micromolar after 24 h of incubation.

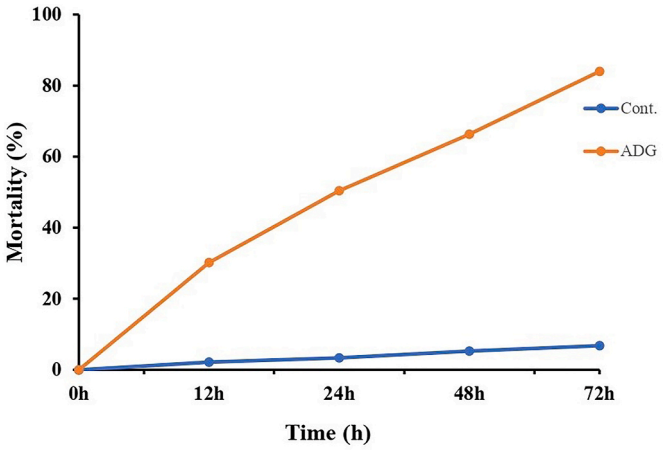

Fig. 2. The effect of ADG on *Giardia* trophozoites. Trophozoites were treated with IC_{50} concentrations of ADG for various time points (0, 12, 24, 48, 72 h). The experiment was conducted in triplicate and the results were evaluated using trypan blue assay.

with IC_{50} concentration of ADG exhibited significant alterations. In Fig. 3A, a control cell containing healthy *Giardia* trophozoites is depicted, showing their normal shape and size, with visible flagella. In contrast, Fig. 3B illustrates the impact of ADG treatment on the cells, leading to shrinkage and the rupture of the trophozoites cell membrane.

3.3. DNA damages

The study investigated the impact of ADG on *Giardia* trophozoites and its potential to induce DNA damage. The results in Fig. 4B revealed that treatment with ADG at IC_{50} concentration led to observable as indicated by a positive signal nucleus damage in *Giardia* trophozoites. In contrast, non-treated control trophozoites showed no evidence of nucleus damage (Fig. 4A). Furthermore, DNA degradation is a characteristic feature associated with apoptotic cell death (Martínez-Espinosa et al., 2015).

3.4. Cell cycle arrest

ADG treatment of trophozoite cells resulted in significant cell cycle arrest, as observed from the PI binding. The fluorescence intensity detected in apoptotic cells, which corresponds to fragmented DNA at G0/G1 phases of cell cycle. This region is commonly referred to as the sub G0/G1 peak. The sub G0/G1 region exhibited a marked and significant increase the amount of apoptotic cells as the duration of ADG treatment. Quantitative analysis of our results (Fig. 5) revealed that the S phase, control cells showed approximately 68% at the start, 50.4% at 12 h, 36.4% at 24 h and 6.3% at 48 h. In contrast, in the G0/G1 phase, control cells displayed a distribution of 14.8% at the beginning, 38.8% at 12 h, 44.9% at 24 h and 20.7% at 48 h. Notably, at the 48-h time point, a significant population of cells, comprising 69.5%, had arrested in the Sub G0 phase. These findings indicate a significant cell cycle arrest induced by ADG treatment.

3.5. ROS generation

The study shown the effects of ADG treatment on oxidative stress generation and ROS production. We observed that trophozoites treated with ADG exhibited significantly higher fluorescence intensity compared to non-treated trophozoites (Fig. 6A, &6B). The result indicate an absence of ROS generation in untreated cells, whereas a notable increase in ROS generation is observed in cells treated with ADG.

3.6. Gene expression changes

We examined the expression profiles of seven genes by measuring their corresponding mRNA fold change levels by RT-qPCR after the treatment of active compound ADG. We have observed the expression of those genes in different time interval. We have shown that (Fig. 7) the expression of arginine deiminase, NADH, and PKC gradually decreased to almost half of its normal expression at 3 h but significantly decreased at 6 and 9 h. Additionally, the expression of other genes, such as FtsJ, cathepsin, cysteine-rich membrane, and surface protein-regulating genes were significantly and gradually decreased at 3, 6, and 9 h. Across various time intervals after treatment with ADG, the expression of seven pathogenic factor-regulating genes were mostly downregulated.

4. Discussion

The anti-cancer and immuno-stimulatory properties of bioactive compounds derived from *A. paniculata* have gained significant attention in recent years. Numerous reports have focused on the ability of ADG, one of these compounds to induce cellular apoptosis in various cell lines (Kumar et al., 2004; Sukardiman et al., 2007). Notably, a recent study explored the cytotoxic effects of ADG on human oral epidermoid carcinoma cell Meng-1 (OEC-M1), revealing its impact on cellular viability, morphology and migration (Liao et al., 2022). Moreover, in this study we examined the in vitro inhibitory effects of ADG on the proliferation and cell death of *Giardia* trophozoites, demonstrating a time-dependent

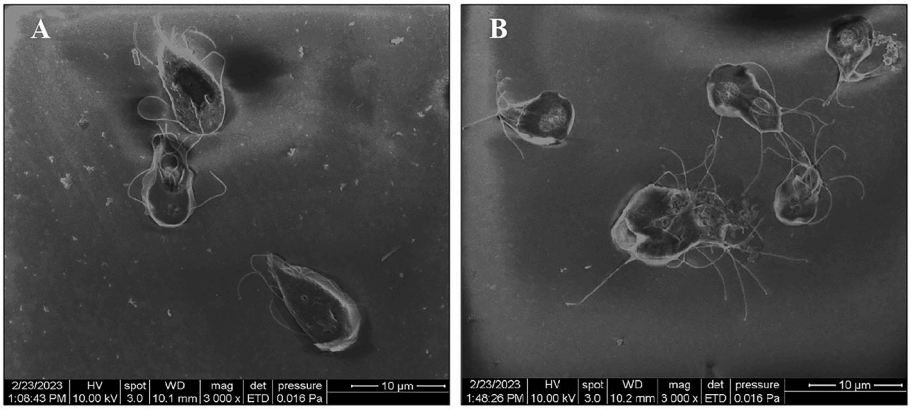

Fig. 3. The figure shows a Scanning Electron Microscopy (SEM) image of *Giardia lamblia* trophozoites after being treated with the IC_{50} concentration of ADG for 24 h. Panel A shows untreated cells, where normal *Giardia* trophozoites are observed, and no changes in morphology are evident. In contrast, Panel B shows ADG-treated cells where trophozoite morphology has changed, and cell membrane rupture is observed.

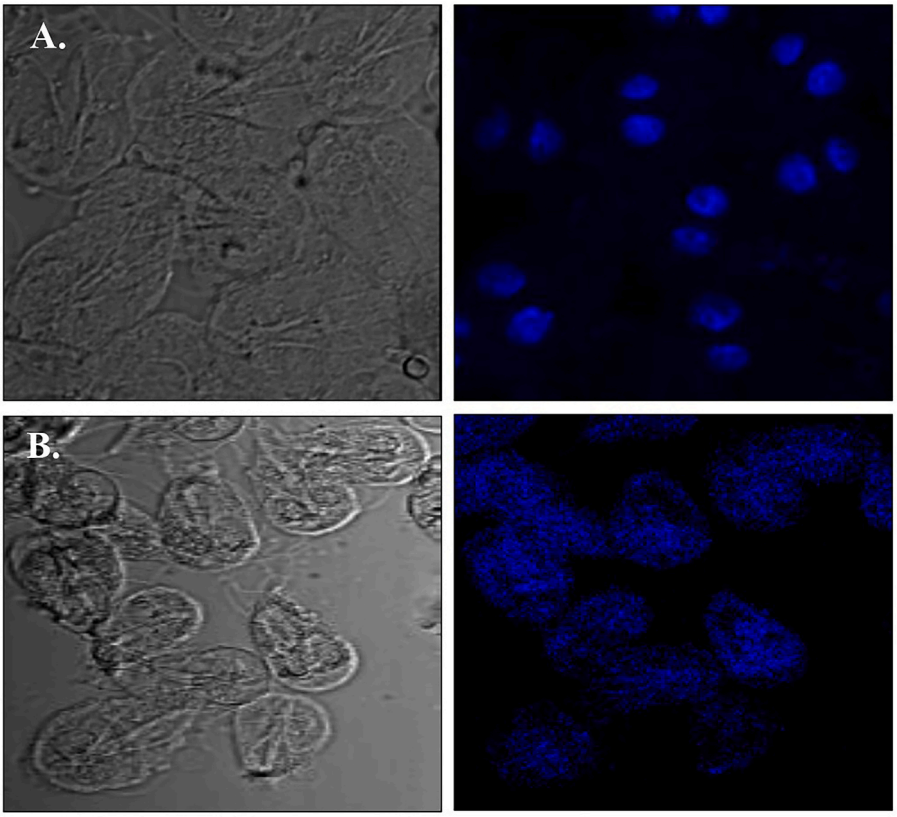

Fig. 4. The figure shows the results of a DNA degradation analysis (A–B). In control living cells where no DNA degradation was observed (A). However, there was significant DNA damage observed after being treated with ADG (B).

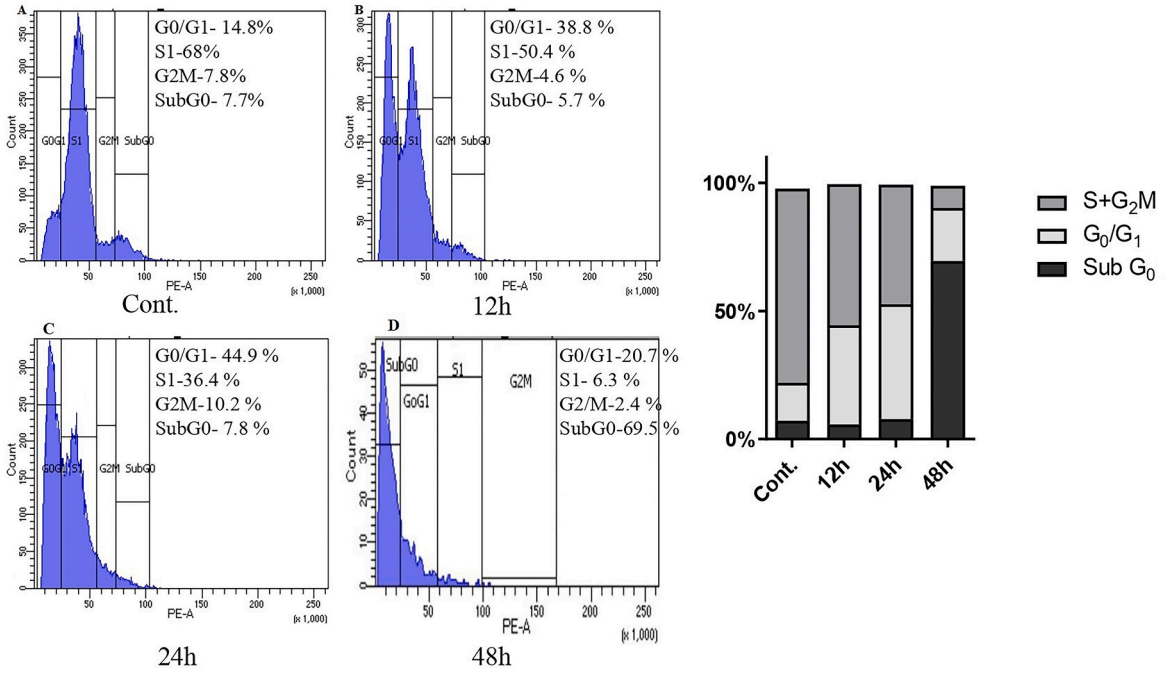

Fig. 5. The effect of ADG treatment on the cell cycle of *Giardia* trophozoites using flow cytometry analysis: Control cells showing the trophozoites normal DNA content in S phase (A). Cells were exposed to the IC_{50} concentration of the ADG and incubated for different time points: 12h (B), 24h (C), and 48h (D). The ADG treatment caused cell cycle arrest, leading to a significant arrested the cells in G0/G1 and subG0 phase.

and dose-dependent relationship (Figs. 1 and 2). The findings reveal that ADG exerts a strong inhibitory effect on the survival of trophozoites with an IC_{50} concentration observed after 24 h of treatment. Moreover, it induces cellular apoptosis-like cell death, characterized by alterations in cellular morphology, DNA damages and the generation of ROS.

In an earlier studied the effects of ADG on PC-3 prostate carcinoma

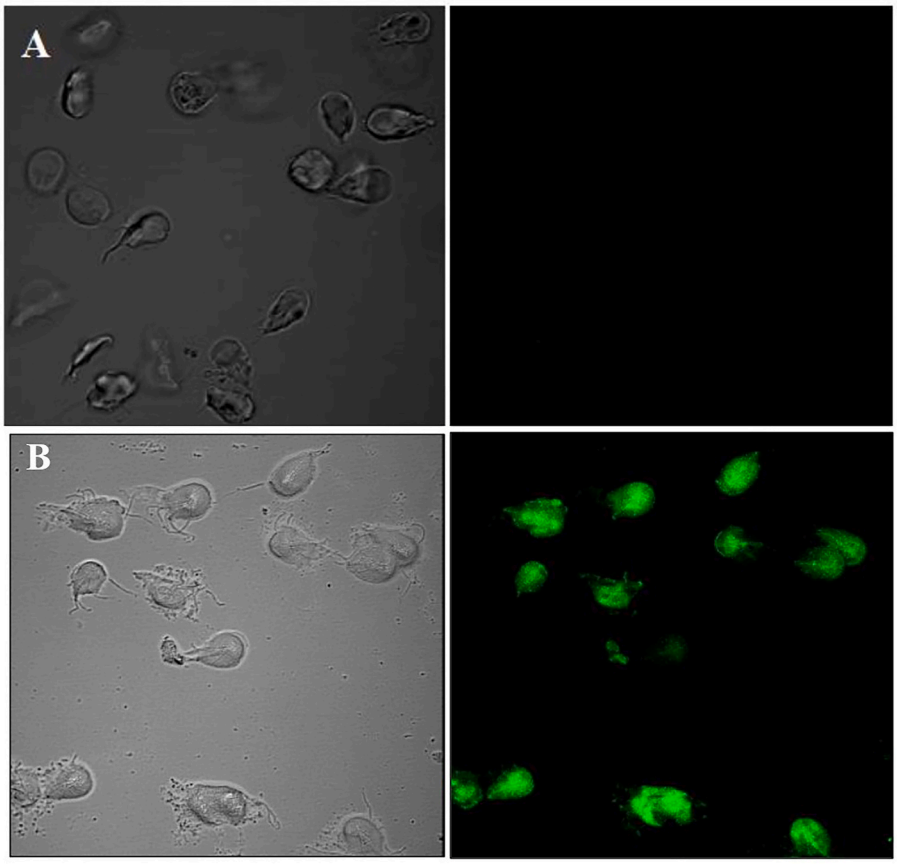

Fig. 6. ROS generation in *Giardia* trophozoites. (A) Control group no ROS generation is observed. (B) ROS generation is enhanced following incubated with ADG.

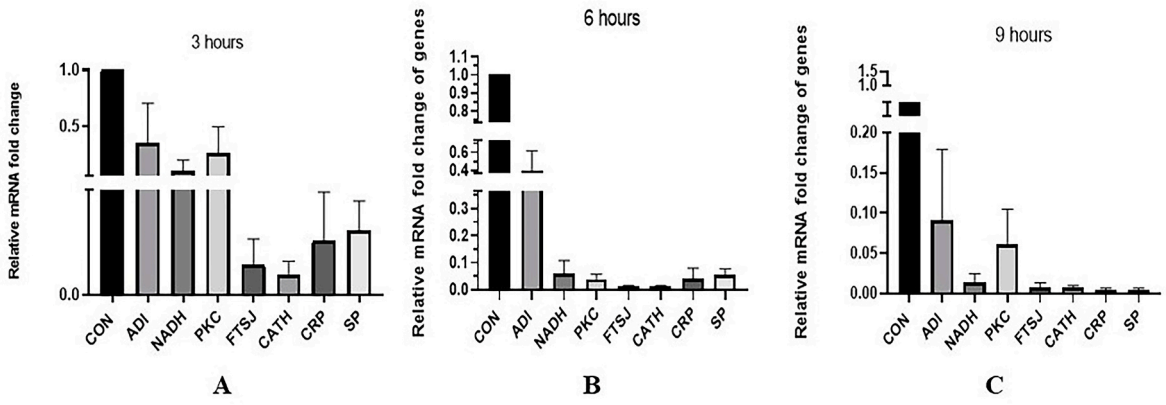

Fig. 7. The effect of ADG on gene expression in *Giardia* trophozoites, the mRNA fold changes using qRT-PCR at different time points: 3 h (A), 6 h (B), and 9 h (C). The genes included ADI (Arginine deiminase), NADH (NADH oxidase), PKC (Protein kinase C), FTSJ (FtsJ cell division protein), CATH (Cathepsin B), CRP (Cysteine-rich membrane protein), and SP (Surface protein).

cells were examined revealing significant morphological and biochemical changes (Kim et al., 2005). ADG has also been found to possess cytotoxic activity against KB cells (human epidermoid carcinoma) and P388 cells (lymphocytic leukemia). The immunostimulatory and anticancer properties of ADG have been observed across various human cancer cell lines (Kumar et al., 2004). In our research, scanning electron microscopy images showed that the ADG led to remarkable changes in morphology of the trophozoites (Fig. 3). These structures are crucial for the attachment of protozoans to the intestinal cell surface. The study suggests that factors beyond microscopic observations, such as metabolic or morphological changes played a role in affecting the adhesion ability of the trophozoites.

ROS are molecules with high reactivity that can result in oxidative stress and damage the cellular structures. Studies have indicated that ADG has the ability to stimulate the production of ROS in different organisms, including cancer cells (Banerjee et al., 2017). Notably, in certain instances, ROS can exhibit antimicrobial properties by interfering with cellular processes and causing damage to DNA and proteins (Vatansever et al., 2013). The excessive generation of ROS and RNS can deplete intracellular antioxidant compounds, ultimately resulting in cell death (Ghosh et al., 2009). Our findings demonstrated that exposure to ADG led to the production of excessive intracellular ROS in *Giardia*

trophozoites (Fig. 6). Notably, ROS were detected, suggesting that oxidative stress induced by ADG could contribute to the cytotoxic effects of this compound in *Giardia* trophozoites. In future investigations it is crucial to identify the specific ROS involved and elucidate the mechanisms responsible for their formation. Recent studies have revealed the ability of certain drugs such as metronidazole, to induce damage to DNA in *Giardia* through in vitro experiments (Uzlikova and Nohynkova, 2014). Similarly, other redox-active drugs like benzimidazole and hydroxymethylnitrofurazone have been reported to primarily affect DNA, a characteristic often associated with necrosis (Davies et al., 2014). When *Giardia* trophozoites were exposed to ADG, DNA degradation was observed, indicating that DNA is the molecule most susceptible to damage (Fig. 4). Specifically, treatment with ADG resulted in cellular damage and oxidative stress in the parasites. While these observations suggest that ADG acts as a DNA-damaging agent, it is important to note that the cytotoxic effects of ADG treatment are multifaceted, as indicated by other research findings. Furthermore, we have demonstrated that the treatment of *Giardia* trophozoites with ADG leads to a rapid decrease in cell adherence, ultimately leading to cell death.

The DNA damage is a molecular occurrence that is strongly linked to the arrest of the cell cycle and the initiation of cell death. In this particular investigation, the administration of ADG led to a reduction of cells in the S/G2M phase. This decrease can be attributed to the interference of ADG with the normal progression of the cell cycle, specifically inhibiting the transition from the S phase to the G2/M phase. Therefore, mitosis is arrested and the cell cycle comes to a halt at the S phase, ultimately triggering cellular apoptosis (Ma et al., 2012; Hung et al., 1996). Our research findings align with previous studies that have demonstrated an apoptotic-like process in *Giardia* when exposed to oxidative stress inducers and metronidazole (Bagchi et al., 2012). Furthermore, multiple studies have provided evidence that ADG is capable of inducing cell cycle arrest predominantly at the G0/G1 stage in a wide range of cancer cells (Geethangili et al., 2008). However, our study presents a novel finding by demonstrating cell cycle arrest at the sub G0/G1 phase specifically in *Giardia* trophozoites upon treatment with ADG (Fig. 5). Further research needs to better understand the underlying mechanisms responsible for cell cycle arrest in ADG-treated trophozoites.

ADG compound under investigation has shown potential as a target for metabolic enzyme, phosphatase and kinase coding, cell division and cell cycle regulatory and surface protein-coding genes. The downregulation of all the seven genes like arginine deiminase, NADH oxidase, Protein kinase c, FtsJ, cysteine protease, cysteine rich membrane protein, and surface protein has been observed at various time points (3h, 6h, and 9h) following ADG treatment (Fig. 7). However, as time progresses, the gene experiences downregulation, indicating a potential shift in cellular requirements or regulatory mechanisms. The cysteine protease and cathepsin B precursor gene have been identified as significant virulence factors and their downregulation has been observed. Additionally, *Giardia* surface protein genes are known to facilitate host cell attachment and colonization, while also potentially protecting the trophozoite from oxygen toxicity (Adam R. D. 2001). However, different time intervals were considered, the seven gene expression was subsequently downregulated. This dynamic regulation of gene expression suggests a complex interplay between ADG and virulence factors. The downregulation of genes implies that ADG may have an effect on *Giardia*, initially activating certain factors and ultimately suppressing their expression at long time's interval.

5. Conclusion

ADG has demonstrated potent inhibitory activity against *Giardia* trophozoites at various doses and time intervals. It also triggers the generation of ROS, leading to oxidative stress. The oxidative stress primarily damages DNA, leading to double-strand breaks and inhibition of cell cycle regulation, ultimately resulting in cell death. ADG treatment also alters the expression of virulent genes. These findings provide potential targets for future research and drug development against *Giardia*, considering the parasites exposure to oxidative stress in the gut. Targeting excessive ROS generation could be a promising strategy for developing new anti-*Giardia* drugs.

Ethical approval

Not Applicable.

Consent for publication

Not Applicable.

Availability of data and materials

The dataset utilized and/or analyzed during the current study has been included in the manuscript. Further datasets are not accessible from the authors.

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CRediT authorship contribution statement

Tapas Haldar: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Sanjib K. Sardar:** Methodology, Formal analysis, Data curation. **Ajanta Ghosal:** Methodology. **Akash Prasad:** Methodology. **Yumiko Saito Nakano:** Investigation. **Shanta Dutta:** Project administration. **Tomoyoshi Nozaki:** Validation. **Sandipan Ganguly:** Writing – review & editing, Validation, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors affirm that they do not have any competing interests related to the publication of the article.

Data availability

Data will be made available on request.

Acknowledgments

Not Applicable.

Abbreviations

ADG	Andrographolide
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
DAPI	4′, 6-diamidino-2-phenylindole
DCFDA	2′-7′-Dichlorodihydrofluorescein diacetate
SEM	Scanning electron microscope
PI	Propidium iodide

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Parasitology

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Genetic characterization of the *Entamoeba moshkovskii* population based on different potential genetic markers

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Research Article

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amoebapore C; chitinase; *Entamoeba moshkovskii*; KERP1; linkage disequilibrium; multilocus sequence typing

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Abstract

Entamoeba moshkovskii, according to recent studies, appears to exert a more significant impact on diarrhoeal infections than previously believed. The efficient identification and genetic characterization of *E. moshkovskii* isolates from endemic areas worldwide are crucial for understanding the impact of parasite genomes on amoebic infections. In this study, we employed a multilocus sequence typing system to characterize *E. moshkovskii* isolates, with the aim of assessing the role of genetic variation in the pathogenic potential of *E. moshkovskii*. We incorporated 3 potential genetic markers: KERP1, a protein rich in lysine and glutamic acid; amoebapore C (apc) and chitinase. Sequencing was attempted for all target loci in 68 positive *E. moshkovskii* samples, and successfully sequenced a total of 33 samples for all 3 loci. The analysis revealed 17 distinct genotypes, labelled M1–M17, across the tested samples when combining all loci. Notably, genotype M1 demonstrated a statistically significant association with diarrhoeal incidence within *E. moshkovskii* infection ($P = 0.0394$). This suggests that M1 may represent a pathogenic strain with the highest potential for causing diarrhoeal symptoms. Additionally, we have identified a few single-nucleotide polymorphisms in the studied loci that can be utilized as genetic markers for recognizing the most potentially pathogenic *E. moshkovskii* isolates. In our genetic diversity study, the apc locus demonstrated the highest H_d value and π value, indicating its pivotal role in reflecting the evolutionary history and adaptation of the *E. moshkovskii* population. Furthermore, analyses of linkage disequilibrium and recombination within the *E. moshkovskii* population suggested that the apc locus could play a crucial role in determining the virulence of *E. moshkovskii*.

Introduction

Amoebic infection is a complex issue as several species are morphologically indistinguishable from each other, including *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba bangladeshi* and *Entamoeba moshkovskii* (Fotedar *et al*., 2007). This makes it challenging to accurately estimate the prevalence of each species and its potential to cause disease in humans. The cysts of a non-pathogenic amoeba, *Entamoeba hartmanni*, can also be mistaken with the pathogenic *E. histolytica* under a microscope (Burrows, 1959) adds an uncertainty. While *E. histolytica* is known to cause pathogenicity in amoebic infections, the actual prevalence of this species is likely overestimated due to these morphological overlaps. Recent research has indicated that *E. moshkovskii* might have a more significant impact on human infections than previously believed. This species has been detected in multiple countries, including the United States, Italy, Iran, Turkey, Indonesia, Colombia, Bangladesh, India, Kenya, Australia, Malaysia, Tanzania, Tunisia and Brazil (Ali *et al*., 2003; Fotedar *et al*., 2007; Khairnar and Parija, 2007; Ayed *et al*., 2008; Beck *et al*., 2008; Delialioglu *et al*., 2008; Anuar *et al*., 2012; Ngui *et al*., 2012; Shimokawa *et al*., 2012; Fonseca *et al*., 2016; Al-Areeqi *et al*., 2017; Kyany'a *et al*., 2019). In addition, *E. moshkovskii* has been identified in farm animals such as pigs, showcasing its potential for zoonotic transmission (Sardar *et al*., 2022). Moreover, it has been documented in non-human primates as well (Levecke *et al*., 2010). A study conducted in eastern India by Sardar *et al*. (2023*a*, 2023*b*) also revealed that *E. moshkovskii* is one of the causative agents of diarrhoeal incidents in humans. The research found that many patients suffering from diarrhoea, infected with *E. moshkovskii*, tested negative for other common enteric pathogens such as bacteria and viruses (Sardar *et al*., 2023*a*, 2023*b*). These findings, combined with various studies conducted in different regions, suggest the potential pathogenicity of *E. moshkovskii* in humans. Therefore, diagnosing diarrhoeal patients should include consideration of *E. moshkovskii* as a potential pathogen to ensure accurate identification of the causative agent. Neglecting this can result in undetermined cases of diarrhoeal illness, leading to improper drug treatments for patients.

Parasitology Research
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RESEARCH

Genotyping and epidemiological distribution of diarrhea-causing isolates of *Giardia duodenalis* in southeastern part of West Bengal, India

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Abstract

The prevalence and genetic diversity of the protozoan pathogen *Giardia duodenalis* have been extensively studied worldwide. There is currently a lack of data regarding the genetic variability of the organism in eastern India. Understanding the circulating genotypes and associated risk factors is crucial for effective planning and implementing control measures. Therefore, the objective of the study was to conduct an epidemiological study to determine the prevalence and identify the various genotypes present. This survey adds to our knowledge on the occurrence and distribution of *Giardia* genotypes in the studied region. The overall prevalence was found to be 6.8%. This parasitic infection was significantly associated with two age groups, i.e., >0–5 years and >5–12 years. Using a multilocus genotyping method, we genotyped 52 human *Giardia* isolates that were obtained from diarrheal patients. Two distinct assemblages were found in the population—30.8% belonged to assemblage A; 63.5% belonged to assemblage B, prevalent in the population; and 5.7% belonged to a combined assemblage A+B. Sub-assemblage AII was found in 17.3% of the cases, followed by sub-assemblage AI (13.5%). High levels of genetic diversity were found within the population of assemblage B undergoing balancing selection. Overall, the high prevalence of the parasite observed, particularly among children, raises a major concern and necessitates implementation of robust control measures. Furthermore, we report the presence of numerous unique genotypes, circulating in this limited geographical boundary, which can be useful dataset for future studies.

Keywords *Giardia* · MLG · SNP · Balancing selection · West Bengal · India

Introduction

Enteric parasites contribute significantly to the burden of diarrheal diseases worldwide. While parasitic diseases run rife in developing countries, sporadic cases and outbreaks are also reported in developed countries (Fletcher et al. 2012). *Giardia duodenalis* (formerly known as *G. lamblia* or *G. intestinalis*) is a non-invasive intestinal protozoan which is commonly associated with diarrheal illnesses (namely giardiasis) in humans, livestock, and wild animals (Takumi et al. 2012). About 280 million cases are reported annually (Lane and Lloyd 2002; Squire and Ryan 2017), with more than 2.5 million annual diarrhea cases coming from developing countries (Thompson 2000). Infection is initiated in the host when the ingested cysts excyst into trophozoites in the duodenum (proximal part of the small intestine) after passing through the acidic environment of the stomach. The trophozoites replicate and remain adhered on the mucosal surface of the intestine, where it causes malabsorption resulting in diarrheal symptoms. Exposure to biliary fluid causes trophozoites to turn into the resistant "cyst" form in the jejunum, which are then shed with the feces to the environment, thereby completing the transmission cycle via the fecal-oral route (Adam 2001).

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RESEARCH

Investigating genetic polymorphism in *E. histolytica* isolates with distinct clinical phenotypes

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Abstract

Amoebiasis is an infection caused by enteric protozoa, most commonly *Entamoeba histolytica*, and is globally considered a potentially severe and life-threatening condition. To understand the impact of the parasite genome on disease outcomes, it is important to study the genomes of infecting strains in areas with high disease prevalence. These studies aim to establish correlations between parasite genotypes and the clinical presentation of amoebiasis. We employ a strain typing approach that utilizes multiple loci, including SREHP and three polymorphic non-coding loci (tRNA-linked array N-K2 and loci 1-2 and 5-6), for high-resolution analysis. Distinct clinical phenotype isolates underwent amplification and sequencing of studied loci. The nucleotide sequences were analysed using Tandem Repeats Finder to detect short tandem repeats (STRs). These patterns were combined to assign a genotype, and the correlation between clinical phenotypes and repetitive patterns was statistically evaluated. This study found significant polymorphism in the size and number of PCR fragments at SREHP and 5-6 locus, while the 1-2 locus and NK2 locus showed variations in PCR product sizes. Out of 41 genotypes, two (I6 and I41) were significantly associated with their respective disease outcomes and were found in multiple isolates. We observed that I6 was linked with a symptomatic outcome, with a statistically significant *p*-value of 0.0183. Additionally, we found that I41 was associated with ALA disease outcome, with a *p*-value of 0.0089. Our study revealed new repeat units not previously reported, unveiling the genetic composition of *E. histolytica* strains in India, associated with distinct disease manifestations.

Keywords Amoebiasis · *Entamoeba histolytica* · Diarrhoea · Amoebic liver abscess · STR

Introduction

Amoebiasis, which is caused by the microaerophilic/anaerobic protozoan parasite *Entamoeba histolytica*, is one of the most severe enteric infections in humans (Das et al. 2014). It is the third leading cause of death due to parasite infection after schistosomiasis and malaria (Singh and Galhotra 2014). The disease can spread via the faecal-oral route and is highly endemic in tropical and subtropical countries with an average socioeconomic status and poor public health (Guillén 2023; Preet et al. 2011). However, only one out of five individuals infected with *E. histolytica* develops intestinal or extraintestinal diseases, and the remaining 80% of infections are asymptomatic (Yanagawa et al. 2020). The specific determinants for this differential outcome of infection are yet to be fully understood, although host defence mechanisms and pathogen virulence are believed to be primary controlling factors. Some studies have reported that host immune responses, such as anti-inflammatory cytokines

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Case Report

First case report of Cyclosporiasis from eastern India: Incidence of *Cyclospora cayetanensis* in a patient with unusual diarrheal symptoms

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Abstract

Cyclospora cayetanensis, a recently described coccidian parasite causes severe gastroenteric disease worldwide. Limited studies are found on the incidence of *C. cayetanensis* infection from India; hence remains largely unknown. To date, no case of cyclosporiasis from eastern India has been reported. In this study, we described an incidental case of *C. cayetanensis* in a 30 years old Bengali female patient with no travel history from eastern India. In June 2022, the patient presented with a history of diarrhoea persisting for more than two months with continuous passage foul smelling stools for which she took multiple antibiotics that were ineffective. There were no Salmonella, Shigella, or Vibrio-like organisms in the patient's faecal sample, and Toxin A/B of Clostridium difficile was also not detected by ELISA. The patient was HIV-negative. Finally, UV autofluorescence and DNA-based diagnosis confirmed the presence of *C. cayetanensis*, and the treatment with a combination of appropriate antibiotics was successful. This case report could raise awareness about *C. cayetanensis* associated diarrhoeal cases in India.

Key words: Cyclosporiasis; *Cyclospora cayetanensis;* diarrhoea; India.

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Background

Cyclospora cayetanensis is a recently described intestinal protozoan parasite belonging to the family *Eimeriidae*, subclass Coccidia, phylum Apicomplexa [1,2]. It was first reported to be an unidentified Isospora-like coccidian parasite causing diarrhoea in patients of Papua New Guinea by Ashford (1979) [2]. Later, in 1994, Ortega, Gilman & Sterling proposed the name *C. cayetanensis* after observing the sporulation and excystation of the organism and the unique morphological features of its oocysts via both light and electron microscopy [2]. Until now, *C. cayetanensis* has been identified solely from the human enteric tract and is now considered an emerging organism, able to cause a severe gastro-enteric disease called cyclosporiasis, especially in immunocompromised individuals, children, and the elderly [2]. Human cyclosporiasis can range from asymptomatic to severe, and if not treated, clinical symptoms can persist for several weeks to a month or more [3,4]. The clinical outcomes are associated with the age and immune response of the host [2,4]. Presently, the biology, risk factors, and routes of transmission of *C. cayetanensis* remain poorly understood [5]. *C. cayetanensis* transmitted to new human hosts via the faecal-oral route or through food and water contaminated by oocysts [6]. Non-sporulated oocysts of *C. cayetanensis* require a maturation period of 7–14 days outside the host body under favourable environmental conditions and thus become infectious [7]. The infective oocysts contain two ovoid sporocysts, each containing two sporozoites. Therefore, the oocysts in fresh stool are non-infectious and it is highly unlikely to be infected by this parasite via fresh stool through faecal-oral contact [8].

Cyclospora is responsible for several outbreaks worldwide in the last two decades, despite having endemicity in only tropical and sub-tropical areas [8]. Although, most of the cases reported in non-endemic

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Prevalence and molecular characterization of *Entamoeba moshkovskii* in diarrheal patients from Eastern India

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Data Availability Statement: Representative sequences obtained in this study were deposited in GenBank under the accession numbers ON965383 - ON965450.

Abstract

Background

Importance of the amphizoic amoeba *Entamoeba moshkovskii* is increasing in the study of amoebiasis as a common human pathogen in some settings. Limited studies are found on the genetic and phylogenetic characterization of *E. moshkovskii* from India; hence remain largely unknown. In this study, we determined the prevalence and characterized the *E. moshkovskii* isolates in eastern India.

Methods

A three-year systemic surveillance study among a total of 6051 diarrhoeal patients from ID Hospital and BC Roy Hospital, Kolkata was conducted for *E. moshkovskii* detection via a nested PCR system targeting 18S rRNA locus. The outer primer set detected the genus *Entamoeba* and the inner primer pair identified the *E. moshkovskii* species. The 18S rRNA locus of the positive samples was sequenced. Genetic and phylogenetic structures were determined using DnaSP.v5 and MEGA-X. GraphPad Prism (v.8.4.2), CA, USA was used to analyze the statistical data.

Result

4.84% (95%CI = 0.0433–0.0541) samples were positive for *Entamoeba* spp and 3.12% (95%CI = 0.027–0.036) were infected with *E. moshkovskii. E. moshkovskii* infection was significantly associated with age groups (X^2 = 26.01, P<0.0001) but not with gender (Fisher's exact test = 0.2548, P<0.05). A unique seasonal pattern was found for *E. moshkovskii* infection. Additionally, 46.56% (95%CI = 0.396–0.537) were sole *E. moshkovskii* infections and significantly associated with diarrheal incidence (X^2 = 335.5,df = 9; P<0.0001). Sequencing

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Research Article OPEN ACCESS

Molecular evidence suggests the occurrence of *Entamoeba moshkovskii* in pigs with zoonotic potential from eastern India

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Abstract: *Entamoeba moshkovskii* Tshalaia, 1941 is prevalent in developing countries and it is considered to be primarily a free-living amoeba, which is morphologically indistinguishable, but biochemically and genetically different from the human infecting, pathogenic *Entamoeba histolytica* Schaudinn, 1903. The pathogenic potential of this organism is still under discussion. *Entamoeba moshkovskii* in human stool samples has been reported in different countries such as the United States, Italy, Australia, Iran, Turkey, Bangladesh, India (Pondicherry), Indonesia, Colombia, Malaysia, Tunisia, Tanzania and Brazil, but no data are available about the occurrence of *E. moshkovskii* in farm animals. This study provides data on the occurrence of *E. moshkovskii* in pigs in a total of 294 fresh faecal samples collected from five different regions in Kolkata, West Bengal, India. Stool samples were tested by nested PCR using primers targeting SSU rDNA of *E. moshkovskii.* The amplified PCR products were further confirmed by RFLP technique. Purified nested PCR products were also sequenced and identified via BLAST program run on the NCBI website to confirm species along with their genetic characteristics of the *E. moshkovskii* isolates. Overall 5.4 % samples were identified as *E. moshkovskii* positive. Results of this study demonstrate that swine can host *E. moshkovskii* and should be considered as a potential natural reservoir for *E. moshkovskii*. However, the occurrence of *E. moshkovskii* infection in pigs was not statistically associated with their faecal consistency, sex and developmental stage.

Keywords: PCR, RFLP, host, natural reservoir, intestinal protists, diarrhea

The genus *Entamoeba* Casagrandi et Barbagallo, 1895 consists of at least nine species: *E. histolytica* Schaudinn, 1903, *E. dispar* Brumpt, 1925, *E. moshkovskii* Tshalaia, 1941, *E. bangladeshi* Royer, 2012, *E. coli* (Grassi, 1879), *E. nuttalli* (Catellani, 1908), *E. hartmanni* Prowazek, 1912, *E. polecki* Prowazek, 1912 and *E. chattoni* Levine, 1961, which are able to reside in the human intestinal lumen (Fotedar et al. 2007b, Delialioglu et al. 2008, Ngui et al. 2012). The first three species are the most prevalent and are morphologically similar under light microscope, but have different biochemical and genetic characteristics (Verweij et al. 2003). Although *E. polecki* has rarely been implicated as a cause of diarrheal disease, it is important to keep in mind that most of species are usually believed as commensal organisms of the human gut except *E. histolytica* and *E. moshkovskii* (see Salaki et al. 1979, Al-Areeqi et al. 2017). *Entamoeba histolytica* is considered as the most recognised pathogen of the human gut. Nonetheless, recent studies have reported the association of *E. moshkovskii* with gastrointestinal clinical manifestations indicating *E. moshkovskii* might be associated with pathogenicity (Ali et al. 2003, Fotedar et al 2007b, Khairnar and Parija 2007, Ngui et al. 2012). So far, the role of *E. moshkovskii* as an etiological agent of diarrhea in humans remains unclear (Clark and Diamond 1991).

Entamoeba moshkovskii was first described as a distinct species from Moscow by Tshalaia in 1941 (see Scaglia et al. 1983). It was primarily considered to be a free-living environmental strain *Entamoeba* sp. and is still regarded as a common protist species found in anoxic sediments and brackish coastal pools. It is osmotolerant in nature and can be cultured in various media suitable for intestinal protists, in which it grows easily at temperatures of 10–15 °C and 37 °C (Diamond and Bartgis 1970, Scaglia et al. 1983, Clark and Diamond 1991).

In 1961, an *E. histolytica*-like strain was obtained from a resident of Laredo, Texas, who suffered from diarrhea, weight loss and epigastric pain; this strain was named as

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