Tropical Biomedicine 41(3): 319-327 (2024)
https://doi.org/10.47665/tb.41.3.013

RESEARCH ARTICLE

Unveiling the anti-giardial properties of *Andrographis paniculata* leaf extract through *in vitro* studies

Haldar, T.[1], Sardar, S.K.[1], Ghosal, A.[1], Das, K.[1,2], Saito-Nakano, Y.[3], Dutta, S.[4], Nozaki, T.[5], Ganguly, S.[1*]

[1]Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED), India
[2]Faculty of Science, Assam Downtown University, Guwahati, 781026, India
[3]Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[4]Division of Bacteriology, ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED), Kolkata, India
[5]Department of Biomedical Chemistry, School of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
*Corresponding author: sandipanganguly@hotmail.com

ARTICLE HISTORY

Received: 30 August 2023
Revised: 5 March 2024
Accepted: 7 March 2024
Published: 30 September 2024

ABSTRACT

Giardiasis, caused by the parasite *Giardia lamblia*, is a prevalent and serious public health concern, particularly affecting children worldwide. The primary mode of transmission for the parasite is through contaminated food and water sources and often leads to the onset of diarrhoea in infected individuals. However, the present medications for Giardiasis treatment often come with numerous side effects, while the growing problem of drug resistance adds a significant complication. Therefore, there is an urgent need for alternative treatments. In this study, we explored the *in-vitro* potential of *Andrographis paniculata* leaf extract as a possible alternative treatment for Giardiasis. Our investigation involved assessing the impact of the ethanolic extract on *Giardia* trophozoites through the analysis of parameters such as cell death, morphological alterations, adherence, ROS generation, and cell cycle dynamics. *A. paniculata* leaf extracts demonstrated significant inhibitory activity against the growth of *Giardia* trophozoites. After being incubated for 24 hours, the test results revealed an IC_{50} value of 51.26µg/ml (95% CI 37.17–65.35) for inducing cell death in *Giardia* trophozoites. We observed a substantial degradation of DNA, alteration in morphology, inhibition in adherence, ROS generation and inhibition of the cell cycle in *Giardia* trophozoites. The findings indicate that *A. paniculata* extract has the potential to be used as a therapeutic treatment for giardiasis. This approach aims to offer a natural therapeutic solution for giardiasis, minimizing side effects and reducing the risk of drug resistance.

Keywords: Giardiasis; diarrhoea; DNA degradation; *Andrographis paniculate*; cell cycle.

INTRODUCTION

Giardiasis is a common intestinal protozoan infection that is widely prevalent in humans (Gardner & Hill, 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 people worldwide are infected with this infection annually. The prevalence rates vary between developing (20-30%) and developed countries (2-5%) (Ankarklev *et al*., 2010). Giardiasis in humans can manifest with a wide range of symptoms, from no noticeable signs to individuals experiencing vomiting, nausea, diarrhoea, and abdominal cramps during the acute phase of the infection. 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 diarrhoea (Merchant & Shroff, 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; Mørch *et al*., 2008). 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 (Alizadeh *et al*., 2006; Savioli *et al*., 2006). Metronidazole, commonly used in the treatment of giardiasis, can lead to various side effects such as vomiting, nausea, lethargy, diarrhoea, loss of appetite, anaemia, 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 (Johnson, 1993; Lemée *et al*., 2000; Voolmann & Boreham, 1993). Furthermore, metronidazole has been associated with reported

carcinogenic, teratogenic and embryogenic properties (Upcroft *et al*., 1999). Given these concerns, it is crucial to investigate and create new treatment options. Examining indigenous traditional medicine and using ethnobotanical knowledge holds great promise for discovering new therapeutic products.

Andrographis paniculata (*Ap*) is one of the most important traditional medicinal plants that is used widely throughout the world. Many countries like India, Bangladesh, China, Pakistan, Hong Kong, Philippines, Thailand, Malaysia and Indonesia use this plant as traditional medicine (Akbar *et al*., 2011; Hossain *et al*., 2014; Jayakumar *et al*., 2013; Kabir *et al*., 2014; Kumar *et al*., 2004). This plant possesses numerous therapeutic properties, including the treatment of diabetes, dysentery, fever, malaria, insect bites and snakebites. (Hossain *et al*., 2014, 2021; Jayakumar *et al*., 2013). 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. It 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) properties.

Our study aims to investigate the antigiardial properties of *A. paniculata* as an alternative treatment.

MATERIALS AND METHODS

Cell culture

G. lamblia Portland1 strain (ATCCR 30888TM) were cultured in TYIS-33 medium supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% adult bovine serum, following the protocol described by Diamond *et al*. (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 35.5°C. After 24 hours of each subculture, cell growth and viability in the culture tubes were examined under a microscope. Trophozoites adhering to the glass tubes indicated their viability. Upon reaching approximately 85–90% confluence in each culture tube, we intended to subculture the tubes to maintain the trophozoite culture.

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 the aqueous extract, 500 g of powder was mixed with 1000 ml of boiled distilled water and subjected to continuous shaking for 120 minutes to ensure thorough mixing. 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 (Harba *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).

Seeding of *Giardia lamblia* trophozoites

The process for collecting trophozoites involved several steps. First, the parasite cultures were subjected to a 10 minutes cold shock to loosen the cells from a glass tube. This was followed by a 5 minutes 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 haemocytometer 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. When a confluent tube culture is divided into three separate tubes, by the third day of subculture, it achieves 80-90% confluence under optimal conditions, including a pH of 6.8 and a temperature of 35.5°C. 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.

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 *G. lamblia* trophozoites during their logarithmic growth phase. In cultures that were nearly 90% confluent, the extracts were treated with various concentrations and durations. The concentrations employed for treatment were 25µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, and 125µg/ml, with a duration of 24 hours. All treated *Ap* extracts were prepared from a stock solution of 10 mg/ml. 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. This experiment was performed by using Annexin V-FITC Kit (BD Bioscience) following manufacturing protocols. After that, 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™ 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.

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 hemocytometer (Sousa *et al*., 2001). This study assessed all three extracts derived from *Ap*.

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.

Cell cycle analysis

The *Giardia* trophozoite cells were collected and washed with PBS (pH-7.2) by centrifuging at 1500 rpm for 10 minutes at 4°C. Then, the cells were dissolved in 70% ethanol and fixed overnight at 4°C (Martínez-Espinosa *et al*., 2015). To avoid cell clumping during fixation, 100µl of 70% ethanol was repeatedly added while vortexing. The Cycle FXCycle™PI/RNase staining solution kit (Thermo Fisher) 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°C following the manufacturer's protocol. Finally, the cell cycle analysis was performed using a flow cytometer (BD FACSAria™ II).

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 (Ordoᴐez-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.

ROS generation

To assess the production of reactive oxygen species (ROS) in trophozoite 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™ II), with an emission wavelength of 517 nm and an excitation wavelength of 492 nm.

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 significant 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.

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 Figure 1 displays the mean values obtained from triplicate measurements using flow cytometry. 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 was 90.21% (Figure 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 hour incubation was determined to be 51.26µg/ml (95% CI 37.17 – 65.35).

Furthermore, 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 22.43% cell death observed at 8 hours and 52.73% at 24 hours

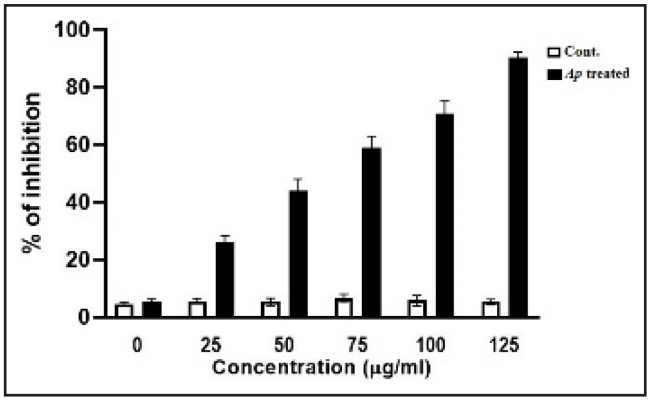

Figure 1. The graph displays the average mortality of *G. lamblia* trophozoites exposed to different concentrations after 24 hours of incubation. The concentrations tested were 25µm/mL, 50µg/ml, 75µm/mL, 100µg/mL, and 125µg/ml. The experiment was conducted in triplicate using a flow cytometry analysis, and the IC_{50} growth inhibition of *Ap* extracts against *Giardia* cells after 24 hours of incubation was calculated to be 51.26µg/ml.

of treatment (as shown in Figure 2). Moreover, at the 72-hour time point, the maximum level of activity was observed, with 97.54% of cells undergoing death after exposure to the IC_{50} concentration.

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 (Figure 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 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.

Cell cycle Arrest

The nuclei of the trophozoites were stained with PI to determine if the plant extract inhibited the cell cycle progression of the treated trophozoites. The results, shown in Figure 5, revealed that the IC_{50} concentration of the extract after different time intervals 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.

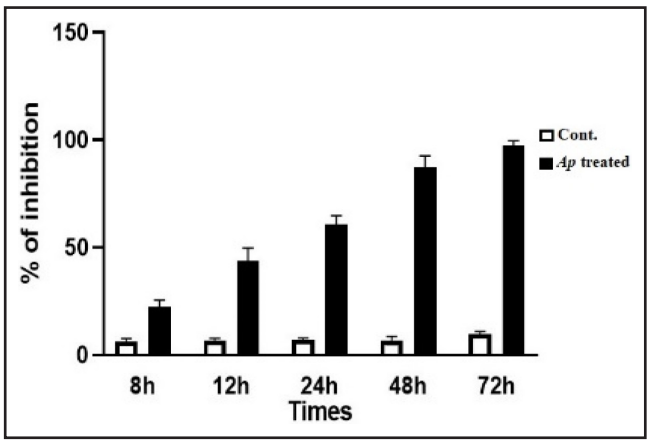

Figure 2. The graph shows the average mortality rate of *G. lamblia* trophozoites that were exposed to IC_{50} concentration for varying time periods ranging from 0-72 hours of incubation. The experiment was carried out in triplicate and the results were analysed using flow cytometry.

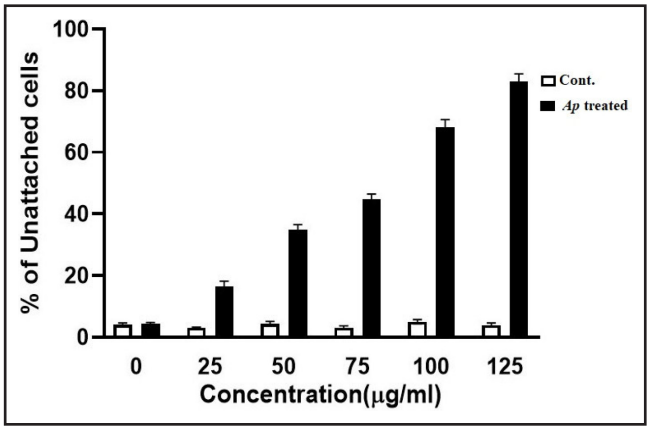

Figure 3. The graph illustrates the mean reduction in adherence of *Giardia* trophozoites after exposure to *A. paniculata* extracts at varying concentrations ranging from 25µg/ml to 125µg/ml. The y-axis represents the percentage of inhibition of adherence, while the x-axis shows the concentration of *Ap* extracts in mg/ml. The graph shows the average of triplicate experiments.

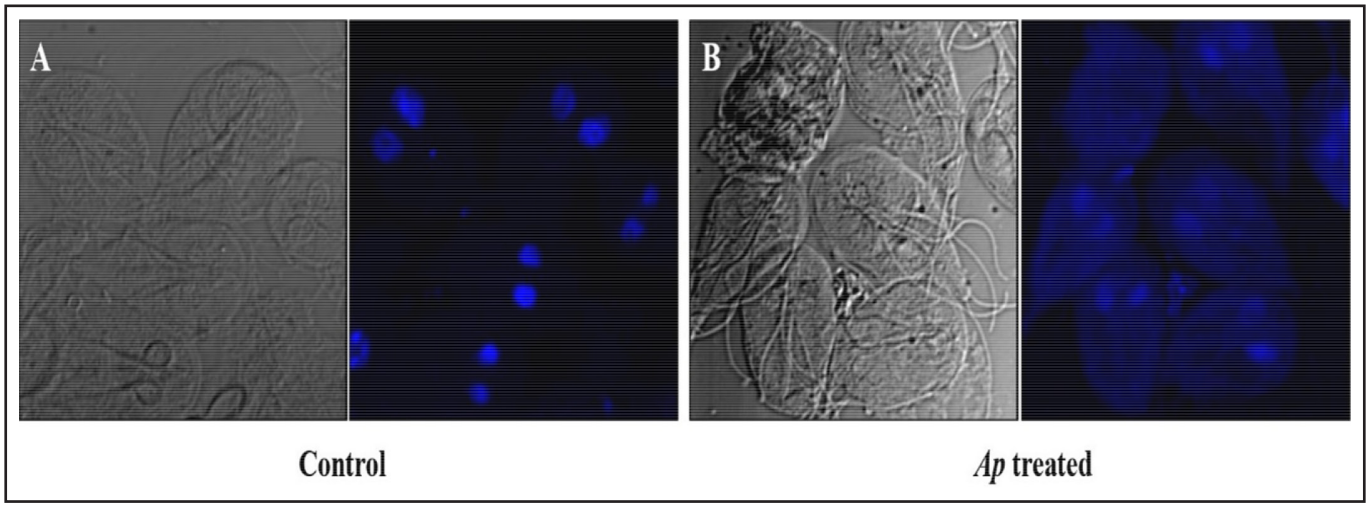

Figure 4. The figure shows the results of a DNA degradation analysis (A-B). In control living cells where no DNA degradation was observed (A). However, there was significant DNA damage observed after being treated with *Ap* extract (B).

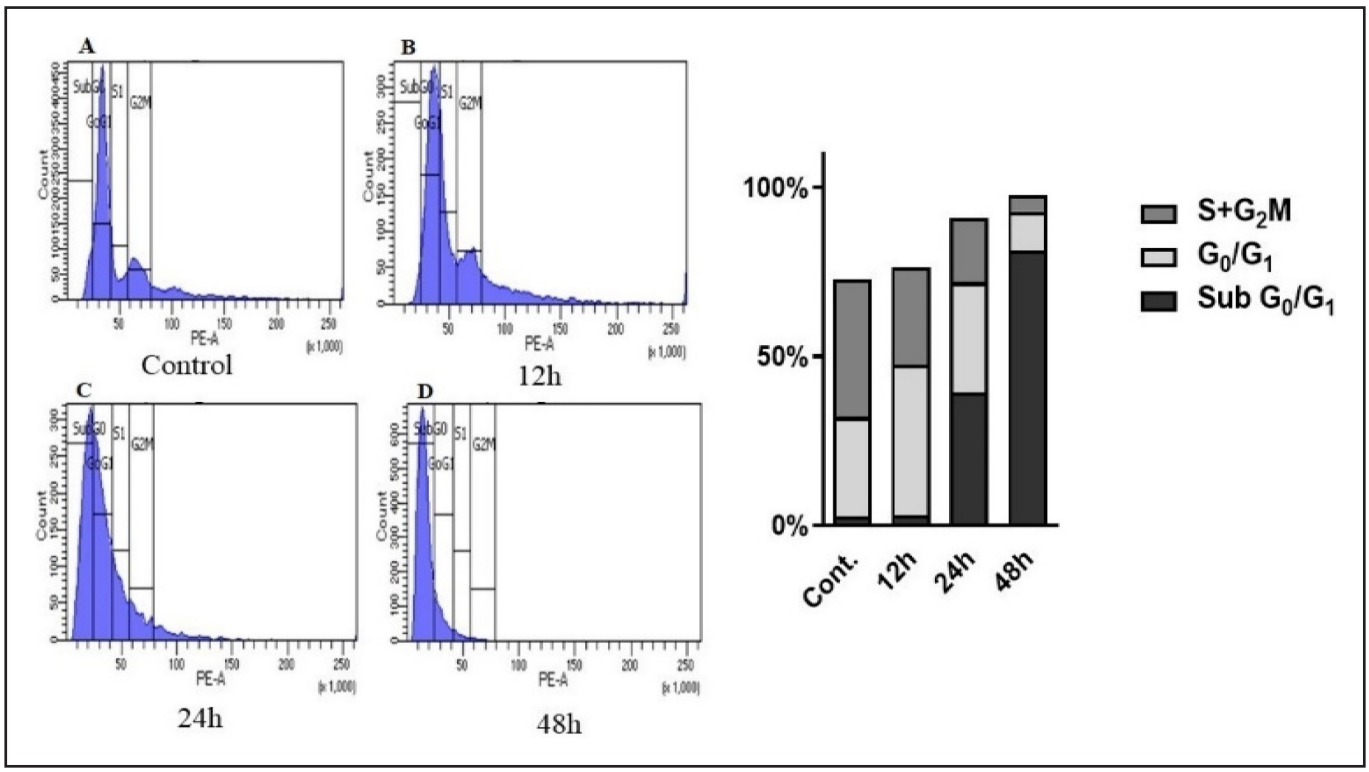

Figure 5. The effect of *Ap* extract treatment on the cell cycle of *Giardia* trophozoites using flow cytometry analysis: Control cells showing the trophozoites contained 4N DNA content in the G1 phase (A). Cells were exposed to the IC_{50} concentration of the extract and incubated for different time 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.

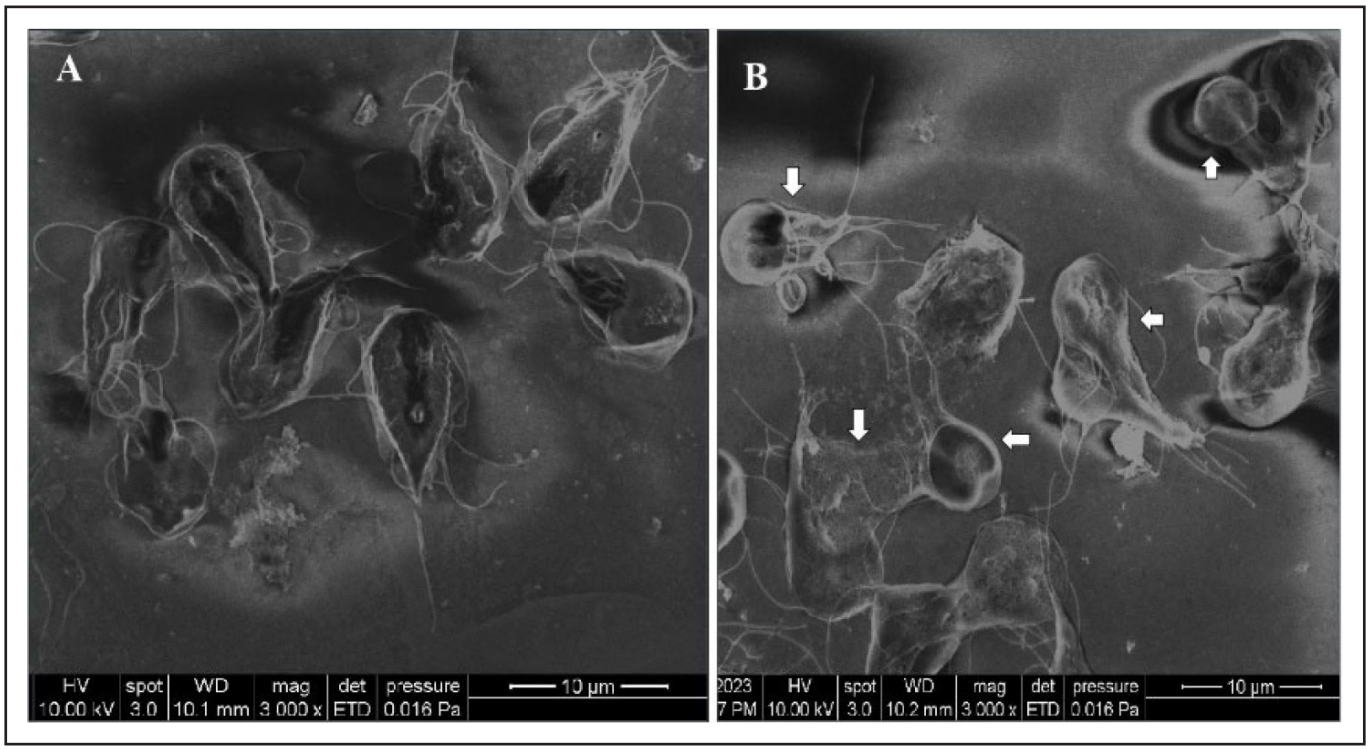

Figure 6. The figure shows a Scanning Electron Microscopy (SEM) image of *Giardia* trophozoites after being treated with the IC_{50} concentration of *Ap* extract for 24 hours. Panel A shows untreated cells, where normal *Giardia* trophozoites are observed, and no changes in morphology are evident. In contrast, Panel B shows extract-treated cells where trophozoite morphology has changed, and cell membrane rupture is also observed.

Cell morphology study

We observed that the shape and size of *Giardia* cells could be altered after the ethanolic extract treatment. Figure 6A shows a control cell with healthy *Giardia* trophozoites displaying normal shape and size, while Figure 6B shows the cells treated with plant extract, resulting in shrinkage and rupture of the cell membrane of the trophozoites. These morphological changes can occur through various mechanisms such as interference with the cell membrane,

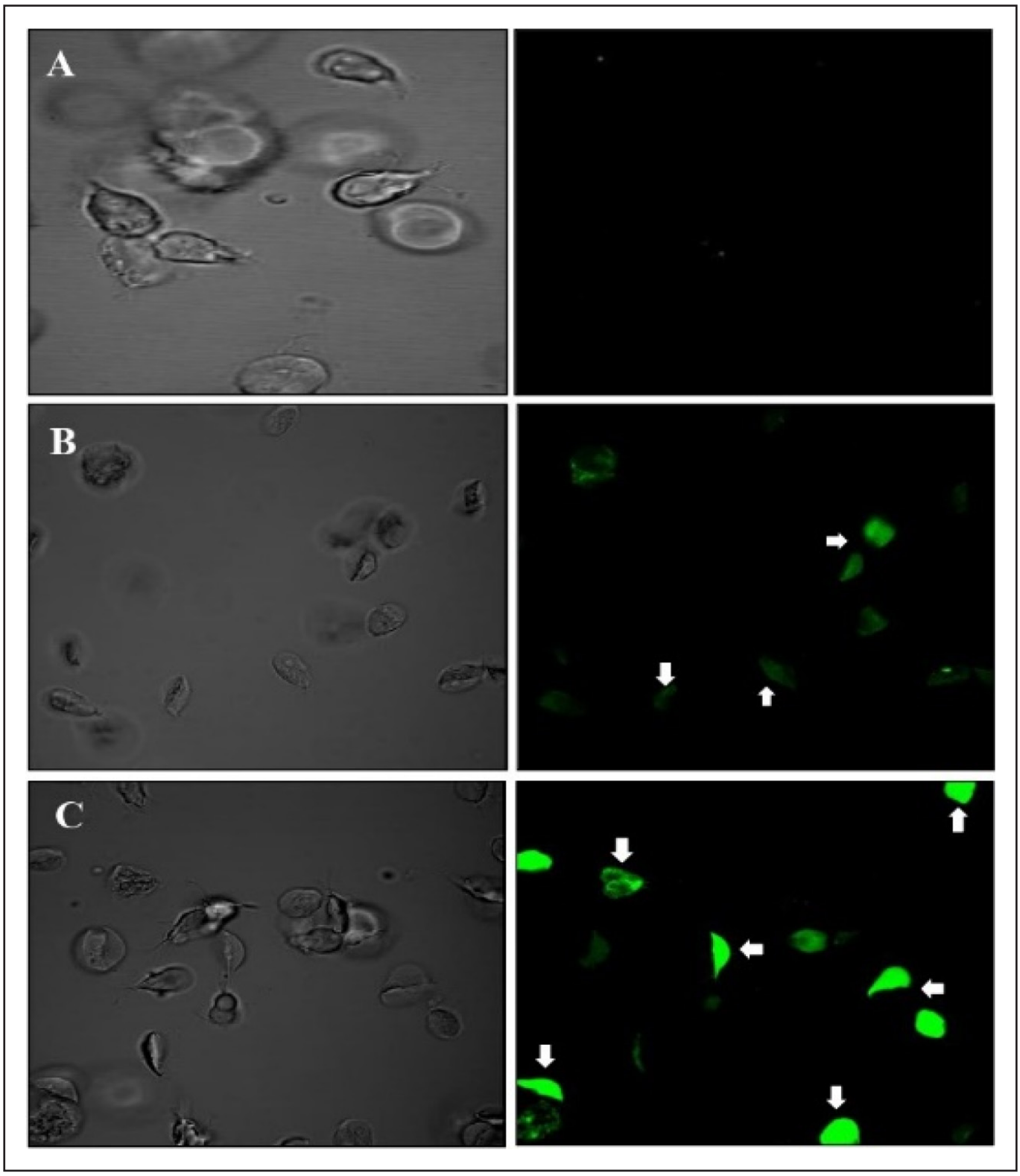

Figure 7. Reactive Oxygen Species (ROS) generation in *Giardia* trophozoites. A) Control group no ROS generation is observed. B) ROS generation is observed after incubated with H2O2. C) ROS generation is enhanced following incubated with *Ap* extract.

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. 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.

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 showed an increase in ROS generation with longer extract exposure, as indicated by rising DCF fluorescence levels (Figure 7C). In the negative control

group, no significant ROS generation was observed; however, the positive control group treated with H2O2 exhibited considerable ROS generation.

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 widespread 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*. 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 anti-*Giardia* 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 (Alnomasy *et al*., 2021), 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 (Hossain *et al*., 2014). It should be noted that the IC_{50} of each plant extract was evaluated under varying laboratory setups and conditions.

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. Our 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 (Ordoñez-Quiroz *et al*., 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 anti-giardia 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 *A. paiculata* 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.

CONCLUSION

The *Ap* extract demonstrates potent inhibitory effects on *Giardia* trophozoites at various doses and durations of exposure. It induces ROS production, causing oxidative stress that damages DNA, leading to cell death by disrupting cell cycle regulation. These findings suggest potential targets for future research and drug development against *Giardia*.

ACKNOWLEDGEMENT

This study was funded by the Indian Council of Medical Research (ICMR), Government of India, Japan Agency for Medical Research and Development (AMED, grant number: JP23fk0108683), Council of Science and Industrial Research (CSIR), Govt. of India and National Institute of Infectious Diseases (NIID), Japan.

Conflict of Interest

The authors declares that they have no conflict of interests.

Authors' contributions

Tapas Haldar: Conceptualization, Visualization, Methodology, Data curation, Writing original draft, Formal analysis; **Sanjib K. Sardar**: Methodology, Formal analysis, Data curation; **Ajanta Ghosal**: Methodology; **Koushik Das**: Methodology; **Yumiko Saito Nakano:** Investigation; **Shanta Dutta:** Project administration; **Tomoyoshi Nozaki:** Validation; **Sandipan Ganguly:** Conceptualization, Validation, Funding acquisition, Review and editing, Supervision.

REFERENCE

Abd-Elhamid, T.H., Abdel-Rahman, I.A.M., Mahmoud, A.R., Allemailem, K.S., Almatroudi, A., Fouad, S.S., Abdella, O.H., Elshabrawy, H.A. & El-Kady, A.M. (2021). A complementary herbal product for controlling Giardiasis. *Antibiotics* **10**: 477. https://doi.org/10.3390/antibiotics10050477

Adam, R.D. (2001). Biology of *Giardia lamblia*. *Clinical Microbiology Reviews* **14**: 447–475. https://doi.org/10.1128/CMR.14.3.447-475.2001

Akbar, S. (2011). *Andrographis paniculata*: a review of pharmacological activities and clinical effects. *Alternative Medicine Review* **16**: 66-77.

Alizadeh, A., Ranjbar, M., Kashani, K.M., Taheri, M.M. & Bodaghi, M. (2006). Albendazole versus metronidazole in the treatment of patients with giardiasis in the Islamic Republic of Iran. *Eastern Mediterranean Health Journal* **12**: 548-554.

Alnomasy, S., Al-Awsi, G.R.L., Raziani, Y., Albalawi, A.E., Alanazi, A.D., Niazi, M. & Mahmoudvand, H. (2021). Systematic review on medicinal plants used for the treatment of *Giardia* infection. *Saudi Journal of Biological Sciences* **28**: 5391-5402. https://doi.org/10.1016/j.sjbs.2021.05.069

Ankarklev, J., Jerlström-Hultqvist, J., Ringqvist, E., Troell, K. & Svärd, S.G. (2010). Behind the smile: cell biology and disease mechanisms of *Giardia* species. *Nature Reviews Microbiology* **8**: 413-422. https://doi.org/10.1038/nrmicro2317

Brandelli, C.L., Giordani, R.B., De Carli, G.A. & Tasca, T. (2009). Indigenous traditional medicine: in vitro anti-giardial activity of plants used in the treatment of diarrhoea. *Parasitology Research* **104**: 1345-1349. https://doi.org/10.1007/s00436-009-1330-3

Carvalho, T.B., Oliveira-Sequeira, T.C. & Guimarães, S. (2014). *In vitro* antigiardial activity of the cysteine protease inhibitor E-64. *Revista do Instituto de Medicina Tropical de Sao Paulo* **56**: 43-47. https://doi.org/10.1590/S0036-46652014000100006

Diamond, L.S., Harlow, D.R. & Cunnick, C.C. (1978). A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. *Transactions of the Royal Society of Tropical Medicine and Hygiene* ***72***: 431-432. https://doi.org/10.1016/0035-9203(78)90144-x

Dyab, A.K., Yones, D.A., Ibraheim, Z.Z. & Hassan, T.M. (2016). Anti-giardial therapeutic potential of dichloromethane extracts of *Zingiber officinale* and *Curcuma longa* in vitro and in vivo. *Parasitology Research* **115**: 2637-2645. https://doi.org/10.1007/s00436-016-5010-9

Escobedo, A.A., Almirall, P., Hanevik, K., Cimerman, S., Rodríguez-Morales, A.J., Almanza, C. & Auza-Santivañez, J. (2018). Giardiasis: a diagnosis that should be considered regardless of the setting. *Epidemiology and Infection* **146**: 1216-1218. https://doi.org/10.1017/S0950268818001504

Frontera, L.S., Moyano, S., Quassollo, G., Lanfredi-Rangel, A., Ropolo, A.S. & Touz, M.C. (2018). Lactoferrin and lactoferricin endocytosis halt *Giardia* cell growth and prevent infective cyst production. *Scientific Reports* **8**: 18020. https://doi.org/10.1038/s41598-018-36563-1

Gardner, T.B. & Hill, D.R. (2001). Treatment of giardiasis. *Clinical Microbiology Reviews* **14**: 114-128. https://doi.org/10.1128/CMR.14.1.114-128.2001

Ghosh, E., Ghosh, A., Ghosh, A.N., Nozaki, T. & Ganguly, S. (2009). Oxidative stress-induced cell cycle blockage and a protease-independent programmed cell death in microaerophilic *Giardia lamblia*. *Drug Design, Development and Therapy* **3**: 103-110. https://doi.org/10.2147/dddt.s5270

Golami, S., Rahimi-Esboei, B., Mousavi, P., Marhaba, Z., Youssefi, M.R. & Rahimi, M.T. (2016). Survey on efficacy of chloroformic extract of *Artemisia annua* against *Giardia lamblia* trophozoite and cyst in vitro. *Journal of Parasitic Diseases* **40**: 88-92. https://doi.org/10.1007/s12639-014-0453-3

Gupta, S., Choudhry, M.A., Yadava, J.N.S., Srivastava, V. & Tandon, J.S. (1990). Antidiarrhoeal activity of diterpenes of *Andrographis paniculata* (Kal-Megh) against *Escherichia coli* enterotoxin in *in vivo* models. *International Journal of Crude Drug Research* **28**: 273-283. https://doi.org/10.3109/13880209009082833

Harba, N.M., Khair, N.S. & Azzaz, M.F. (2019). Anti-giardial activity of the ethanolic extracts of *Cymbopogon citratus* leaves (Lemongrass) and *Pulicaria undulata* herb in comparison with Metronidazole, *in vitro* and *in vivo*. *Egyptian Journal of Medical Microbiology* **28**: 33-42. https://doi.org/10.21608/ejmm.2019.282965

Hossain, M.S., Urbi, Z., Sule, A. & Hafizur Rahman, K.M. (2014). *Andrographis paniculata* (Burm. f.) Wall. ex Nees: a review of ethnobotany, phytochemistry, and pharmacology. *The Scientific World Journal* **2014**: 274905. https://doi.org/10.1155/2014/274905

Hossain, S., Urbi, Z., Karuniawati, H., Mohiuddin, R.B., Moh Qrimida, A., Allzrag, A.M.M., Ming, L.C., Pagano, E. & Capasso, R. (2021). *Andrographis paniculata* (burm. f.) wall. ex nees: an updated review of phytochemistry, antimicrobial pharmacology, and clinical safety and efficacy. *Life* **11**: 348. https://doi.org/10.3390/life11040348

Hotez, P.J., Brooker, S., Bethony, J.M., Bottazzi, M.E., Loukas, A. & Xiao, S. (2004). Hookworm infection. *The New England Journal of Medicine* **351**: 799-807. https://doi.org/10.1056/NEJMra032492

Jayakumar, T., Hsieh, C.Y., Lee, J.J. & Sheu, J.R. (2013). Experimental and clinical pharmacology of *Andrographis paniculata* and its major bioactive phytoconstituent andrographolide. *Evidence-Based Complementary and Alternative Medicine* **2013**: 846740. https://doi.org/10.1155/2013/846740

Johnson, P.J. (1993). Metronidazole and drug resistance. *Parasitology Today* **9**: 183-186. https://doi.org/10.1016/0169-4758(93)90143-4

Kabir, M.H., Hasan, N., Rahman, M.M., Rahman, M.A., Khan, J.A., Hoque, N.T., Bhuiyan, M.R., Mou, S.M., Jahan, R. & Rahmatullah, M. (2014). A survey of medicinal plants used by the Deb barma clan of the Tripura tribe of Moulvibazar district, Bangladesh. *Journal of Ethnobiology and Ethnomedicine* **10**: 19. https://doi.org/10.1186/1746-4269-10-19

Kumar, R.A., Sridevi, K., Kumar, N.V., Nanduri, S. & Rajagopal, S. (2004) Anticancer and immunostimulatory compounds from *Andrographis paniculata*. *Journal of Ethnopharmacology* **92**: 291-295. https://doi.org/10.1016/j.jep.2004.03.004

Lemée, V., Zaharia, I., Nevez, G., Rabodonirina, M., Brasseur, P., Ballet, J.J. & Favennec, L. (2000). Metronidazole and albendazole susceptibility of 11 clinical isolates of *Giardia duodenalis* from France. *The Journal of Antimicrobial Chemotherapy* **46**: 819-821. https://doi.org/10.1093/jac/46.5.819

Martínez-Espinosa, R., Argüello-García, R., Saavedra, E. & Ortega-Pierres, G. (2015). Albendazole induces oxidative stress and DNA damage in the parasitic protozoan *Giardia duodenalis*. *Frontiers in Microbiology* **6**: 800. https://doi.org/10.3389/fmicb.2015.00800

Merchant, R.H. & Shroff, R.C. (1996). The prevalence of HIV infection in children with extensive tuberculosis and chronic diarrhoea. *AIDS Asia* **3**: 21-23.

Mishra, U.S., Mishra, A., Kumari, R., Murthy, P.N. & Naik, B.S. (2009). Antibacterial activity of ethanol extract of *Andrographis paniculata*. *Indian Journal of Pharmaceutical Sciences* **71**: 436-438. https://doi.org/10.4103/0250-474X.57294

Misra, P., Pal, N.L., Guru, P.Y., Katiyar, J.C., Srivastava, V. & Tandon, J.S. (1992). Antimalarial activity of *Andrographis paniculata* (Kalmegh) against *Plasmodium berghei* NK 65 in *Mastomys natalensis*. *International Journal of Pharmacognosy* **30**: 263-274. https://doi.org/10.3109/13880209209054010

Mørch, K., Hanevik, K., Robertson, L.J., Strand, E.A. & Langeland, N. (2008). Treatment-ladder and genetic characterisation of parasites in refractory giardiasis after an outbreak in Norway. *The Journal of Infection* **56**: 268-273. https://doi.org/10.1016/j.jinf.2008.01.013

Nanduri, S., Nyavanandi, V.K., Thunuguntla, S.S., Kasu, S., Pallerla, M.K., Ram, P.S., Rajagopal, S., Kumar, R.A., Ramanujam, R., Babu, J.M. *et al*. (2004). Synthesis and structure-activity relationships of andrographolide analogues as novel cytotoxic agents. *Bioorganic & Medicinal Chemistry Letters* **14**: 4711-4717. https://doi.org/10.1016/j.bmcl.2004.06.090

Okhuarobo, A., Falodun, J.E., Erharuyi, O., Imieje, V., Falodun, A. & Langer, P. (2014). Harnessing the medicinal properties of *Andrographis paniculata* for diseases and beyond: a review of its phytochemistry and pharmacology. *Asian Pacific Journal of Tropical Disease* **4**: 213-222. https://doi.org/10.1016/S2222-1808(14)60509-0

Ordoñez-Quiroz, A., Ortega-Pierres, M.G., Bazán-Tejeda, M.L. & Bermúdez-Cruz, R.M. (2018). DNA damage induced by metronidazole in *Giardia duodenalis* triggers a DNA homologous recombination response. *Experimental Parasitology* **194**: 24-31. https://doi.org/10.1016/j.exppara.2018.09.004

Pekthong, D., Martin, H., Abadie, C., Bonet, A., Heyd, B., Mantion, G. & Richert, L. (2008). Differential inhibition of rat and human hepatic cytochrome P450 by *Andrographis paniculata* extract and andrographolide. *Journal of Ethnopharmacology* **115**: 432-440. https://doi.org/10.1016/j.jep.2007.10.013

Raj, D., Ghosh, E., Mukherjee, A.K., Nozaki, T. & Ganguly, S. (2014). Differential gene expression in *Giardia lamblia* under oxidative stress: significance in eukaryotic evolution. *Gene* **535**: 131-139. https://doi.org/10.1016/j.gene.2013.11.048

Savioli, L., Smith, H. & Thompson, A. (2006). *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. *Trends in Parasitology* **22**: 203-208. https://doi.org/10.1016/j.pt.2006.02.015

Sousa, M.C., Gonnalves, C.A., Bairos, V.A. & Poiares-Da-Silva, J. (2001). Adherence of *Giardia lamblia* trophozoites to Int-407 human intestinal cells. *Clinical and Diagnostic Laboratory Immunology* **8**: 258-265. https://doi.org/10.1128/CDLI.8.2.258-265.2001

Upcroft, J.A., Campbell, R.W., Benakli, K., Upcroft, P. & Vanelle, P. (1999). Efficacy of new 5-nitroimidazoles against metronidazole-susceptible and -resistant *Giardia, Trichomonas*, and *Entamoeba* spp. *Antimicrobial Agents and Chemotherapy* **43**: 73-76. https://doi.org/10.1128/AAC.43.1.73

Voolmann, T. & Boreham, P. (1993). Metronidazole resistant *Trichomonas vaginalis* in Brisbane. *The Medical Journal of Australia* **159**: 490. https://doi.org/10.5694/j.1326-5377.1993.tb137978.x

Xu, Y., Tang, D., Wang, J., Wei, H. & Gao, J. (2019). Neuroprotection of Andrographolide against microglia-mediated inflammatory injury and oxidative damage in PC12 neurons. *Neurochemical Research* **44**: 2619-2630. https://doi.org/10.1007/s11064-019-02883-5