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Understanding methodologies applied to *Giardia lamblia* and *Trichomonas vaginalis*: from isolation to *in vitro* screening

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Understanding methodologies applied to *Giardia lamblia* and *Trichomonas vaginalis*: from isolation to *in vitro* screening

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Resumo

As infeções causadas por parasitas protozoários continuam a representar um problema de saúde pública, destacando-se a giardíase, uma parasitose intestinal, e a tricomoníase, uma doença sexualmente transmissível. O metronidazol permanece como o *gold standard* no tratamento de ambas as parasitoses. Contudo, o número crescente de casos de resistência registados e os efeitos secundários dos fármacos justificam a necessidade de descobrir novos compostos com potencial terapêutico. As plantas medicinais constituem uma fonte valiosa de novos compostos terapêuticos devido às diversas atividades biológicas dos seus constituintes fitoquímicos.

O presente trabalho teve como principal objetivo avaliar o potencial antiparasitário de plantas medicinais selecionadas em trofozoítos de *Giardia lamblia* e *Trichomonas vaginalis*. Para tal, foram realizadas duas revisões sistemáticas, aplicando a metodologia Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020), sobre técnicas utilizadas ao longo do ciclo de vida de *G. lamblia* e sobre ensaios de viabilidade celular utilizados para avaliar a atividade antigiardia e antrichomonas de compostos com potencial terapêutico. Além disso, foram realizados ensaios laboratoriais para a determinação do potencial antiparasitário de extratos aquosos e metanólicos de plantas medicinais selecionadas com base em estudos etnobotânicos realizados em Portugal.

Palavras-chave: Etnobotânica; *Giardia lamblia*; *in vitro screening*; plantas medicinais; *Trichomonas vaginalis*.

Abstract

Protozoan parasitic infections continue to represent a public health problem, with giardiasis, an intestinal parasitosis, and trichomoniasis, a sexually transmitted disease, being particularly noteworthy. Metronidazole remains the gold standard for treating both parasitic diseases. However, the increasing number of recorded resistance cases and the side effects of these drug highlight the need to discover new compounds with therapeutic potential. Medicinal plants constitute a valuable source of new therapeutic compounds due to the diverse biological activities of their phytochemical constituents.

The main objective of this study was to evaluate the antiparasitic potential of selected medicinal plants on trophozoites of *Giardia lamblia* and *Trichomonas vaginalis*. To achieve this goal, two systematic reviews were conducted, employing the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) methodology, on techniques used throughout the life cycle of *G. lamblia* and viability assays used to evaluate the antigiardial and antitrichomonal activity of compounds with therapeutic potential. Furthermore, experimental assays were conducted to determine the antiparasitic potential of aqueous and methanolic extracts of medicinal plants selected based on ethnobotanical studies carried out in Portugal.

Keywords: ethnobotany; *Giardia lamblia*; *in vitro* screening; medicinal plants; *Trichomonas vaginalis*.

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List of Abbreviations

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

$CaCl_2$ – Calcium chloride

CDC – Centers for Disease Control and Prevention

CDW – Cold Distilled Water

CGSM – Cell Growth in Semi-solid Medium

DMSO – Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

EC_{50} - Half Maximal Effective Concentration

ECDC – European Centre for Disease Prevention and Control

ECP – Ether Clarification Procedure

EU – European Union

FACS – Fluorescence Activated Cell Sorting

FDA – Fluorescein diacetate

FDA/PI – Fluorescein Diacetate/Propidium Iodide

GI – Growth Inhibition

HBSS – Hank's Balanced Salt Solution

HCl – Hydrogen chloride

HIV – human immunodeficiency virus

IC_{50} – Half Maximal Inhibitory Concentration

ID_{50} - Median Infective Dose

IMS – Immunomagnetic Separation

INT - p-iodonitrotetrazolium violet

KCl – Potassium chloride

MIC - Minimum Inhibitory Concentration

MLC - Minimum Lethal Concentration

MMSM – Macrodilution Method in Semi-solid Medium

MSS – Magnetic Separation System

MTS/PMS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/Phenazine Methosulfate

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaCl – Sodium chloride

$NaHCO_3$ – Sodium bicarbonate

NaOH – Sodium hydroxide

NPR – 4-nitrophenyl b-dribofuranoside

OD – Optical Density

PAHO – Pan American Health Organization

PBS – Phosphate-buffered saline

PBST – PBS with 0.01% Tween 20

PI – Propidium Iodide

PRISMA 2020 – Preferred Reporting Items for Systematic Reviews and Meta-Analyses

RNA – Ribonucleic acid

SCLM – Subculture in Liquid Medium

SFM – Sucrose Flotation Method

SPIDER – Sample, Phenomenon of Interest, Design, Evaluation, Research type

SPSS – Statistical Software Package for the Social Science

SYBR Green I – 2-[4-(2-hydroxyethyl)-1-piperazinyl]-N,N-dimethyl-6-(4-nitrophenylamino)-3-hydroxy-2,7-naphthalenedisulfonamide

UK – United Kingdom

USA – United States of America

WHO – World Health Organization

XTT – 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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1. Introduction

Given recent advances in the medical field and efforts to improve public health, the prevalence of parasitic diseases has been decreasing. However, in developing countries and tropical regions, parasitoses continue to be a public health problem (Lee et al., 2019; Sutrave & Richter, 2021). Protozoan parasitic infections remain a major public health concern due to their substantial socioeconomic impact and high morbidity and mortality globally, despite recent advances in prevention and treatment (Custodio, 2016). Among these, infections caused by *Giardia lamblia* and *Trichomonas vaginalis* are endemic in Portugal.

G. lamblia (also known as *G. duodenalis* or *G. intestinalis*) is a flagellate parasite that causes giardiasis, a major cause of epidemic or sporadic diarrhoea worldwide (Adam, 2021). The infection rate can reach 30% in developing countries due to poor sanitation and limited water treatment facilities, and 7% in developed countries, where it is the most common cause of parasitic diarrhoea (Ahmed, 2023; Lee et al., 2019). Giardiasis is listed by the World Health Organization (WHO) as a neglected disease that endangers human health (Savioli et al., 2006). In 2019, 18.004 confirmed cases were reported in the European Union and European Economic Area. In Portugal, 45 cases per 100 000 habitants were reported in 2019 (European Centre for Disease Prevention and Control (EDCD), 2022).

The clinical manifestations of giardiasis range from asymptomatic to symptomatic, with symptoms occurring more frequently in children (Adam, 2021; Das et al., 2023). Symptoms may be acute or chronic and include diarrhoea, abdominal cramps, and other gastrointestinal symptoms (Lee et al., 2019). Chronic giardiasis may lead to malabsorption and weight loss (Das et al., 2023). Dehydration and electrolyte imbalance are significant risks, especially for young children in developing countries (Leung et al., 2019).

G. lamblia has a life cycle with two stages: an infectious cyst stage and a proliferating trophozoite stage (Fink et al., 2020). The infection is transmitted via the faecal-oral route and results from the ingestion of cysts through the consumption of water or food contaminated with faeces, through person-to-person transmission and, rarely, through animal-to-person transmission (Leung et al., 2019). After ingestion, excystation occurs in the duodenum, where the cysts are exposed to gastric acid, bile, and trypsin. Each cyst gives origin to two motile trophozoites through nuclear division (Adam, 2021). Clinical symptoms appear during the trophozoite stage (Leung et al., 2019). Trophozoites multiply by binary fission into numerous trophozoites. The detached trophozoites pass down the small intestine into the colon and

encyst. The cysts travel through the colon and are excreted in the faeces, where they are instantly infectious. The cysts are dormant and hardy and can survive in cold water for up to 3 months. If ingested, the cycle starts all over again, with an incubation period ranging from 1 week to 3 weeks (Ahmed, 2023; Fink et al., 2020).

Diagnosis is confirmed by detecting the presence of trophozoites, cysts, or giardia-specific antigen in faecal samples (collected thrice on separate days in a week) or trophozoites in duodenal fluid or duodenal biopsy (Ahmed, 2023; Beer et al., 2017; Custodio, 2016).

Therapy is not routinely administered to asymptomatic individuals (Custodio, 2016). For symptomatic patients, 5-nitroimidazoles such as metronidazole and tinidazole are used (Ahmed, 2023). Typically, 250 mg of metronidazole is administered three times a day for 5 to 7 days, or a single dose of 2 g of tinidazole is administered. Nitazoxanide can also be used, and 500 mg is administered twice daily for 3 days (Beer et al., 2017; The Medical Letter, 2013). In Portugal, albendazole is also approved for the treatment of giardiasis, with 400 mg usually administered once daily for 5 days (Direção Geral de Saúde, 2017).

T. vaginalis is a flagellated protozoan parasite of the human genital tract and is the cause of the most prevalent curable sexually transmitted disease globally, with an estimated 156 million cases per year (Harfouche et al., 2024; WHO, 2024). It predominantly affects women of childbearing age, particularly those of low socioeconomic status and those with poor hygiene (Nwokah et al., 2019). Trichomoniasis has been included in the list of neglected parasitic infections by the Centers for Disease Control and Prevention (CDC) (Cantey et al., 2021).

The clinical manifestations of trichomoniasis are variable and dependent on the sex of the infected individual. Infection of the female genital tract is symptomatic in approximately 50% to 75% of cases and can cause a range of symptoms, including pruritus, local oedema, erythema, dysuria, vaginal discharge and vaginitis. Approximately 80% of infections of the male genital tract are generally asymptomatic; however, mild urethritis, epididymitis or prostatitis may occur (Ibáñez-Escribano & Nogal-Ruiz, 2024; Nwokah et al., 2019).

Trichomoniasis is associated with serious conditions such as prostate cancer, cervical cancer, adverse pregnancy outcomes, and an increased likelihood of human immunodeficiency virus (HIV) infection (Benchimol et al., 2022; Harfouche et al., 2024). *T. vaginalis* infection is also considered a risk marker for other infections such as bacterial vaginosis, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Nwokah et al., 2019).

T. vaginalis does not present a cyst form in its life cycle, existing only in a teardrop-shaped trophozoite form. However, endoflagellar forms have been observed (Benchimol et al., 2022). While nonsexual transmission, such as transmission via fomites, can occur, the parasite is almost exclusively transmitted through sexual contact. The parasite has the ability to survive for up to three hours in a moist environment (Edwards et al., 2014).

The culture of *T. vaginalis* from clinical samples, swab samples taken from the vaginal canal or cervix of female patients, or urethral discharge from male patients, is considered the gold standard for the diagnosis of this organism (Ibáñez-Escribano & Nogal-Ruiz, 2024).

The treatment of trichomoniasis includes a single dose of 2 g of metronidazole or tinidazole (Benchimol et al., 2022; The Medical Letter, 2013).

Recent data on the prevalence of *G. lamblia* and *T. vaginalis* infections are limited and may be underestimated due to various factors. One primary reason is the asymptomatic nature of these infections. In individuals with giardiasis, 50 to 75% of infections are asymptomatic (Leung et al., 2019). Most patients with trichomoniasis are either asymptomatic or mildly symptomatic, with nearly 80% of infected men being asymptomatic (Ibáñez-Escribano & Nogal-Ruiz, 2024; Nwokah et al., 2019; Shafir et al., 2009). Moreover, giardiasis and trichomoniasis are not always notifiable diseases. Particularly, trichomoniasis is not currently a reportable infection in either developed or developing countries, which can further contribute to the lack of recent prevalence data (Edwards et al., 2014; Shafir et al., 2009). In Portugal, only giardiasis is a notifiable disease; however, the latest data refers to November 2018 (Despacho n.º 15385-A/2016, 2016; Serviço Nacional de Saúde, 2018). Furthermore, the lack of standardized diagnostic methods, particularly for identifying resistant and asymptomatic cases, surveillance programs and reporting mechanisms for these infections can hinder the collection of recent prevalence data. Variability in diagnostic techniques, limited access to testing facilities, and inconsistencies in reporting practices across different regions can all contribute to gaps in data on the prevalence of these parasitic infections (Ibáñez-Escribano & Nogal-Ruiz, 2024; Nwokah et al., 2019).

Metronidazole has been the drug of choice for both giardiasis and trichomoniasis. However, there has been an increase in the recognition of metronidazole-resistant strains. Side effects from metronidazole treatment are common and include nausea, vomiting, metallic taste, and dizziness (Benchimol et al., 2022; Solaymani-Mohammadi et al., 2010). Some side effects have been reported in up to 12% of patients (Tiwari et al., 2008). When standard metronidazole

treatment for presumed-resistance fails, alternatives include receiving a higher dose of metronidazole or switching to a related 5-nitroimidazoles. These alternatives are not easily accessible to all patients due to costs and availability within countries (Lam et al., 2023). Even so, these substitute drugs cause different severe side effects that may lead to treatment interruption (Juárez-Saldivar et al., 2023). Resistance to 5-nitroimidazole can be expected in 5–10% of symptomatic patients, and cross-resistance to 5-nitroimidazole drugs has been reported (Ibáñez-Escribano & Nogal-Ruiz, 2024; Lam et al., 2023). The absence of pharmacological alternatives to cope with treatment failure, hypersensitivity to 5-nitroimidazoles, or side effects increases the risk of transmission and the development of chronic infections. Therefore, protozoan infectious diseases are one of the main areas of opportunity in drug discovery due to the lack of effective pharmacological treatment and the increasing number of drug-resistant cases (Hernández-Ochoa et al., 2022; Juárez-Saldivar et al., 2023; Zheng et al., 2014).

Natural resources, particularly plants, are important sources of new bioactive products due to their wide variety and complexity of metabolites with potential therapeutic value (Benlarbi et al., 2023; Neiva et al., 2014). In the past, a large number of antimicrobial compounds were discovered from synthetic and natural products for the treatment and control of infectious agents (Kebede et al., 2021). Notably, most clinically proven pharmaceuticals are derived from plants (Benlarbi et al., 2023; Kebede et al., 2021). Medicinal plants and their derivatives offer valuable sources of new therapeutic agents for treating common human diseases and controlling protozoan parasites and their vectors (Ramirez-Moreno et al., 2017). Ethnobotanical and ethnopharmacological studies have significantly contributed to the discovery of active plant-based products (Neiva et al., 2014). The WHO estimates that 80% of the global population uses plants as alternative therapy, highlighting the effectiveness of traditional medicines (Ramirez-Moreno et al., 2017). For centuries, plants with therapeutic properties have been used in traditional medicine, offering potential solutions with fewer side effects than synthetic alternatives (Das et al., 2010). Medicinal plants are less expensive, less harmful, and often as effective as conventional drugs, making them attractive alternatives for treating parasitic diseases amidst growing concerns over drug resistance (Bahmani et al., 2014). The increasing acceptance of traditional medicine as an alternative healthcare approach, combined with the development of microbial resistance to current antibiotics, underscores the need to further investigate the antimicrobial potential of medicinal plants. This

investigation could help mitigate the side effects associated with synthetic antimicrobial agents while offering a vast, untapped source of pharmaceuticals for treating infectious diseases in both plants and humans (Das et al., 2010). Specific methods for cell culture and maintenance, as well as for evaluating the antiparasitic potential of medicinal plants against *G. lamblia* and *T. vaginalis* are essential for validating the potential of these natural alternatives.

Given the high global prevalence of parasitic infections and the urgent need for new therapeutic options, incorporating an ethnopharmacological approach in research is crucial. This approach not only aids in discovering new treatments but also provides accessible and sustainable therapeutic solutions. Continuous research on *G. lamblia* and *T. vaginalis*, is essential for effective prevention and treatment strategies. Exploring new therapies, particularly those derived from medicinal plants, can address the growing resistance to conventional treatments, thereby improving the global health and quality of life of those affected by these parasitic infections.

The main aim of this dissertation was to evaluate the antiparasitic potential of extracts from selected medicinal plants against *G. lamblia* and *T. vaginalis* trophozoites. To achieve this primary aim, the following specific objectives were defined:

- Develop a systematic review of methodologies used for the isolation and culture of *G. lamblia*;
- Conduct a systematic review of methodologies employed for evaluating antigiardial and antitrichomonal activity;
- Determine the antiparasitic potential of aqueous and methanolic extracts of selected medicinal plants based on ethnobotanical studies conducted in Portugal.

This dissertation is divided into three chapters, each written in an article format corresponding to a specific objective to facilitate the organization and interpretation of information:

- Chapter I systematically reviews the literature on methodologies used throughout the life cycle of *G. lamblia*;
- Chapter II systematically reviews the literature on methodologies for evaluating antigiardial and antitrichomonal activity;
- Chapter III presents the practical component of the study, detailing the *in vitro* experimental tests assessing the activity of extracts from selected medicinal plants against *G. lamblia* and *T. vaginalis* trophozoites.

2. Chapter 1 – Exploring methodologies for *Giardia lamblia* from isolation to excystation: a systematic review

2.1. Abstract

Giardia lamblia is a flagellate protozoan parasite and a major cause of epidemic and sporadic diarrhoea worldwide, known as giardiasis. The clinical and public health burden of giardiasis underscores the need for robust methods to study and manage this pathogen. This study aimed to systematically review the main methodologies cited in the literature for the *G. lamblia* life cycle, including isolation, purification, axenization, excystation and encystation. The guiding research question was: "What are the predominant methodologies employed for the isolation, purification, excystation and encystation of *G. lamblia*?". A systematic literature review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) statement using databases such as MEDLINE, ScienceDirect, and Web of Science; with the following search equation: "("axenization" OR "isolation" OR "excystation" OR "encystation" OR "purification") AND ("method") AND ("giardia")". A total of 39 studies were included in the review; 56 methods for isolation and purification, 7 methods for excystation, and 3 methods for both axenization and encystation of *G. lamblia* were identified. Methods for isolation and purification exhibited significant differences, with filtration, centrifugation, density gradient, and immunomagnetic separation being the most cited methods. Effectiveness differed based on the source and sample type, highlighting the need for standardized protocols to ensure consistent and reliable results. The isolation and purification methods for *G. lamblia* are notably variable and lack uniformity and efficiency compared to the more consistent methods used for other life cycle stages of the parasite.

Keywords: Axenization; encystation; excystation; *Giardia lamblia;* giardiasis; isolation.

2.2. Introduction

The etiological agent of giardiasis, *Giardia lamblia*, is a flagellate protozoan parasite that is a major cause of both epidemic and sporadic diarrhoea worldwide (Ahmed, 2023). Giardiasis is prevalent and has occurred in outbreaks across the globe since the 1970s. It is listed by the World Health Organization (WHO) as a neglected disease that endangers human health (Savioli et al., 2006).

The lifecycle of *G. lamblia* comprises two distinct stages adapted to different environmental conditions: an infectious cyst stage and a proliferating trophozoite stage (Ahmed, 2023; Alvarado et al., 2022; Leung et al., 2019). The infection is mainly transmitted via the faecal–oral route, resulting from the ingestion of cysts through the consumption of water or food contaminated with faecal matter (Ahmed, 2023; Leung et al., 2019). After ingestion, excystation occurs in the duodenum due to exposure to gastric acid, bile and pancreatic proteases, resulting in the release of two motile trophozoites per cyst.

Trophozoites primarily inhabit the proximal small intestine (duodenum and jejunum) and attach to enterocytes via the ventral sucking disc. During the trophozoite stage, clinical symptoms appear as trophozoites replicate by binary fission into numerous trophozoites. Detached trophozoites pass through the small intestine to the colon, where encystation occurs (Ahmed, 2023; Leung et al., 2019). Cysts are excreted in the faeces and are immediately infectious, remaining viable in the environment until ingestion by a new host, consequently restarting the cycle all over again (Alvarado et al., 2022).

Axenic *in vitro* systems enable the study of parasite biochemistry and differentiation mechanisms throughout the parasite life cycle (Savioli et al., 2006). Excystation and encystation are differentiation processes that occur in response to environmental conditions perceived by the parasite in the host. Both processes can be induced by simulating the conditions found in the human gastrointestinal tract (Alvarado et al., 2022; Boucher & Gillin, 1990).

Excystation and encystation are targets for chemotherapeutic and immunotherapeutic intervention (Savioli et al., 2006). The clinical and public health burden of giardiasis underscores the need for robust methods to study and manage this pathogen. Diverse techniques have been developed and refined over the years to isolate *G. lamblia* cysts from environmental samples, maintain axenic cultures under laboratory conditions, and understand the mechanisms underlying encystation and excystation.

The aim of this systematic review was to identify the main methods cited in the literature used throughout the *G. lamblia* life cycle, including isolation, purification, axenization, excystation and encystation.

2.3. Materials and methods

The current systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) statement (Page et al., 2021). The SPIDER tool adapted from Cooke et al. (2012) was employed to formulate the following research question: "What are the predominant methodologies employed for the isolation, purification, excystation, axenization and encystation of *G. lamblia*?" (Table 1).

Table 1 - Description of the employed SPIDER tool adapted from Cooke et al. (2012).

SPIDER Tool	
S – Sample	Studies involving *G. lamblia*
PI – Phenomenon of Interest	Methodologies for isolation, purification, excystation, axenization and encystation
D – Design	Experimental studies
E – Evaluation	Effectiveness, reproducibility
R – Research type	Methodological studies, experimental research

2.3.1. Information sources and search strategy

A literature search was conducted across the MEDLINE, ScienceDirect, and Web of Science databases using the following search equation: "("axenization" OR "isolation" OR "excystation" OR "encystation" OR "purification") AND ("method") AND ("giardia")". In the ScienceDirect and Web of Science databases, the search was based on title, abstract or author-specified keywords. In MEDLINE, the available MeSH terms were used. The databases were accessed on 4 April 2024.

2.3.2. Eligibility criteria

Studies eligible for inclusion were research articles, written in English, Portuguese, or Spanish. These articles were specifically required to assess methods to evaluate the processes of encystation, excystation, isolation, purification and axenization of *G. lamblia.* Studies lacking explicit specifications of the methods employed were excluded, as were reviews and meta-analyses. No restrictions were imposed regarding the timeframe of the search.

2.3.3. Quality assessment

A critical appraisal was used for assessing the quality of the studies included in the review, through the development of a checklist adapted from McConn et al. (2024). The checklist included parameters such as clearly specified criteria for sample selection and collection, mention of the source of the samples used, use of controls to validate the experimental procedures, explanation of the methods used and their adaptation to typical laboratory conditions, adequate description of statistics for study replication, assessment of *G. lamblia* viability and functionality, and appropriate discussion of the obtained results. Eight parameters, in the form of questions, were scored on a yes–no basis and "not applicable or not sure" (Table 2). The articles were grouped by the number of "yes" by quality into a scale: 1–2 unsatisfactory; 3–4 satisfactory; 5–6 good; and 7–8 excellent.

Table 2 – Quality assessment criteria used for the evaluation of the articles adapted from McConn et al. (2024).

Questions	Criteria		
1. Did the paper clearly specify the criteria for sample selection and collection, including any inclusion or exclusion criteria?	Yes	No	Not applicable/not sure
2. Did the paper mention the source of the samples used (clinical isolates (human or animal), reference isolates, water environment (river, surface water))?			
3. Were controls appropriately used to validate experimental procedures?			
4. Did the paper explain the methods used (including isolation, purification, axenization, excystation and encystation)?			
5. Were the methods described in the paper appropriate and adaptable to typical laboratory conditions?			
6. Were the statistics sufficiently described to enable the study to be repeated?			
7. Was the viability and functionality of *G. lamblia* assessed?			
8. Are the results both justified by the methods provided and discussed in the paper's discussion section?			

2.4. Results and Discussion

The electronic search yielded a total of 196 articles, with 85 entries retrieved from PubMed, 61 from ScienceDirect, and 50 from Web of Science (Figure 1). These sets of records were downloaded from each respective database and then integrated into the Rayyan platform (Ouzzani et al., 2016). This consolidation served the dual purpose of removing duplicate records and facilitating the retrieval of pertinent articles. Following the elimination of duplicates, a total of 136 studies remained for further evaluation. The titles and abstracts of all identified studies were independently examined by two reviewers, according to predefined inclusion and exclusion criteria. Disagreements between the researchers were resolved through consensus discussions, obviating the need for a third opinion. Records that were evidently irrelevant were excluded. In cases where the abstract and/or title did not provide sufficient information to comply with the inclusion criteria, the full text of the report was obtained for thorough evaluation. Consequently, 56 studies were selected for full-text reading, and these were independently assessed by the same two reviewers. Articles that did not meet all inclusion criteria after the full-text assessment (n=17) were excluded from further examination. Figure 1 illustrates and summarizes the complete study selection process.

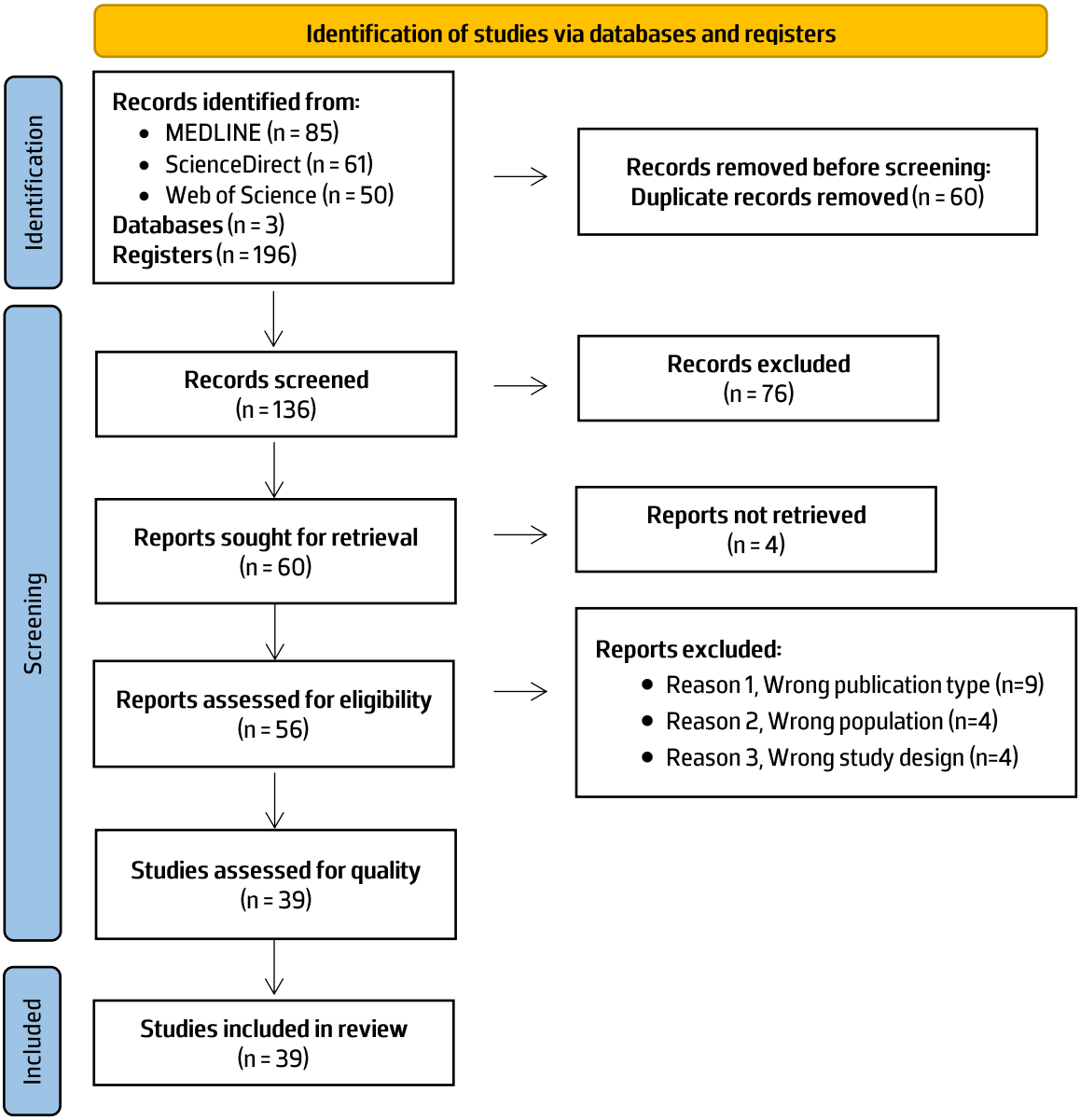

Figure 1 – Flowchart of the selection procedure adapted from the PRISMA 2020 statement (Page et al., 2021).

2.4.1. Quality assessment

To minimize the risk of bias, an assessment of the quality of the articles included in the review was conducted through the development of an 8-question checklist. The studies included in the review (n=39) were evaluated based on this checklist (Figure 2). Of these, 72.5% were classified as good, followed by excellent (17.5%) and satisfactory (10%). No articles were classified as unsatisfactory, and therefore, no articles were excluded.

Article	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Overall
Adaway et al. 2017	✓	✓	?	✓	✓	✗	✗	✓	
Ahmad et al. 1997	?	✓	?	✓	✓	✓	✗	✓	
Al-Tukhi et al. 1991	✓	✓	?	✓	✓	✓	✓	✓	
Alvarado and Wasserman 2006	✓	✓	✗	✓	✓	?	✓	✓	
Barazesh et al. 2011	?	✓	✓	✓	✓	✓	✗	✓	
Bénéré et al. 2007	?	✓	✓	✓	✓	✓	✓	✓	
Bertrand et. al 2004	✗	✓	✓	✓	✓	?	✓	✓	
Bertrand et. al 2009	?	✓	✓	✓	✓	✓	✓	✓	
Bezaglo et al. 2020	✗	✓	✗	✓	✓	✓	✗	✓	
Bielec et al. 1996	?	✓	✓	✓	✓	?	✓	✓	
Castro-Hermida et al. 2008	✗	✓	✗	✓	✓	✓	✓	✓	
Cruz et al. 2003	✓	✓	✓	✓	✓	✓	✓	✓	
de Araújo et al. 2018	?	✓	?	✓	✓	✓	✗	✓	
Dehghani-Samani et al. 2019	?	✓	?	✓	✓	✓	✓	✓	
Douglas et al. 1987	?	✓	✗	✓	?	?	✓	✓	
Feely and Eriandsen 1981	?	✓	✗	✓	✓	✓	✓	✓	
Ferrari et al. 2006	✓	✗	✓	✓	✓	✓	✗	✓	
Ferrari and Veal. 2003	✗	✓	✗	✓	✓	✓	✗	✓	
Gilmour et al. 1991	✗	✓	✗	✓	✓	✗	✓	✓	
Gords et al. 1985	✓	✓	✓	✓	✓	?	✓	✓	
Hsu and Huang 2000	✗	✓	✓	✓	✓	✓	✗	✓	
Isaac-Renton et al. 1992	✓	✓	✓	✓	✓	✓	✓	✓	
Jaime Massanet-Nicolau 2003	✗	✓	✓	✓	✓	✓	✗	✓	
Kane et al. 1991	✗	✓	✓	✓	✓	✓	✓	✓	
Karanis et al. 2006	✗	✓	✗	✓	?	✗	✓	✓	
Keserue et al. 2011	✓	✓	✓	✓	✓	?	✗	✓	
Lyu and Wen 2020	✗	✗	✗	✓	✓	✗	✓	✗	
Neto et al. 2010	✗	✓	✓	✓	✓	✗	✗	✓	
Pinto et al. 2016	✗	✗	✗	✓	✓	✓	✓	✓	
Polverino et al. 2004	✗	✓	✗	✓	✓	✓	✗	✓	
Ramadan et al. 2010	✗	✓	✓	✓	✓	✗	✓	✓	
Roubin et al. 2002	✗	?	✓	✓	✓	✗	✓	✓	
Santos et al. 2004	✗	✓	✓	✓	✓	?	✗	✓	
Sauch et al. 1991	✗	✓	✗	✓	✓	✓	✓	✓	
Siripanth et al. 1995	✗	✓	✗	✓	✓	✓	✗	✓	
Sroka et al. 2013	✗	✓	✗	✓	✓	✓	✗	✓	
Terrones et al. 2019	✓	✓	✗	✓	✓	✓	✗	✓	
Uda-Shimoda et al. 2014	✗	✓	✓	✓	✓	✗	✗	✓	
Zhang et al. 2013	✗	✓	✓	✓	✓	✗	✗	✓	

Caption

✓	Yes	Excellent
?	Not applicable/ not sure	Good
✗	No	Satisfactory
		Unsatisfactory

Figure 2 – Quality assessment of the reviewed articles considering the questions (Q1 to Q8) formulated in Table 2.

2.4.2. Characterization of the included studies

The 39 studies were conducted in 25 different countries, with the majority conducted in Brazil (15%), followed by the United States of America (12.5%). Most of the reviewed studies were experimental. Moreover, the included studies used a wide range of sample types, including water samples (n=20), human faecal samples (n=16), animal faecal samples (n=8), commercial samples (n=11), reference isolates (ATCC 30888 and ATCC 30957; n=5), laboratory strains (n=2) and others (rectal swabs, intestinal scrapings, and duodenal fluid; n=4). The water samples in the included studies were collected from various sources (Table 3).

Table 3 – Water sample sources of the included studies.

Water source		Number of studies
Human consumption		7
River		5
Treatment plant	Raw	6
	Sewage sludge	4
	Treated	3
	Sewage effluent	2
	Secondary effluent	1
Wastewater	Abattoir	2
	Treatment plant	4
Lake/Pond/ Recreational		4
Dam/Spring/Well		3
Ultrapure		2
Marine		1

The present systematic review identified a variety of methods used for the isolation, purification, excystation, axenization, and encystation of *G. lamblia*. Many authors often use the terms "isolation" and "purification" interchangeably when referring to methods for obtaining *G. lamblia* cysts or trophozoites. This underscores the very closely related nature of both processes, where isolation typically refers to the initial separation of the organism from its environment, e.g., using a filtration method, and purification involves subsequent steps to further clean the sample, e.g., the sucrose flotation method. To reflect this common practice and ensure comprehensive coverage, both isolation and purification methods were compiled into the same table (Table 4).

Table 4 - Methods for the isolation and purification of *G. lamblia*.

Citation	Study type	Country	Sample	Method	Description
Feely & Erlandsen (1981)	Experimental	United States of America (USA)	Intestinal scrapings (rats)	Suspension in Hank's balanced salt solution (HBSS)	The scrapings were suspended in 10 ml of HBSS (pH 7.2) and aspirated several times to break up tissue fragments and dislodge trophozoites. The suspension was centrifuged (200g/1min), and the supernatant was further centrifuged (200g/10min) to pellet the trophozoites. The bottom 4 ml of each tube, including the trophozoite-enriched pellet was transferred to a petri dish and incubated at 37°C for 20 min to allow trophozoites to adhere to the dish surface. The suspension was removed, leaving the attached trophozoites undisturbed. The dishes were rinsed twice with warm HBSS (37°C), tilting the dish to allow 1–2 ml of HBSS to wash over the surface before removal. The surface was covered with 2 ml of fresh HBSS (37°C).
				Temperature alternation	Petri dishes with attached trophozoites were initially incubated at 37°C (cycle 1; 37°C) to promote attachment. Subsequently, the dishes were incubated at 4°C for 20 min (cycle 1; 4°C). After this, 2 ml of cold HBSS was used to rinse and collect detached trophozoites, leaving intestinal debris adherent to the dish. The collected washings were transferred to clean petri dishes, and the procedure was repeated for two additional cycles alternating between f 37°C and 4°C to purify the trophozoites. After each cycle, reattached trophozoites were rinsed with 1 to 2 ml of HBSS.
Douglas et al. (1987)			Faecal (human)	Filtration and washing by low-speed centrifugation	A stool sample was diluted 1:20 in cold distilled water (CDW) or phosphate-buffered saline (PBS), filtered through cheesecloth, and chilled in ice-water (4°C). Sephadex G-50 columns were prepared by suspending 30 g of Sephadex G-50 in 1 litre of PBS (pH 7.2) for 3-5 days at room temperature. The diluted stool sample was layered onto the packed Sephadex column, and the cysts were washed with 5 volumes of CDW or PBS. Fractions of 50 ml were collected, and cysts were identified microscopically. Fractions containing cysts were washed 4-5 times in CDW or PBS by centrifugation (1000rpm/5min/4°C).
Al-Tukhi et al. (1991)		Saudi Arabia		Sucrose gradient	Stool samples were shaken with CDW for 30 min and allowed to settle. The supernatant was collected and layered onto a gradient of 0.4 M and 0.85 M sucrose solutions, followed by centrifugation (600g/10min). The material at the water/sucrose interface was aspirated and washed twice with CDW. Subsequently, the cyst pellet was resuspended in a mixture of 5 ml CDW and 7 ml ethyl ether, vortexed, and centrifuged again (600g/10min).

Citation	Study type	Country	Sample	Method	Description
Gilmour et al. (1991)	Comparative	United Kingdom (UK)	Water (tap, raw, recreational and marine) Sewage effluents	Washing machine method	Shredded filters were washed with 0.1% Tween 80 using a customized washing machine. The eluate concentrated to 20 ml, either by centrifugation or sedimentation, and the pellet volume was recorded. Cysts were further concentrated by sucrose density flotation, and the fluid above the pellet, along with the interface, was aspirated. It was then diluted in CDW and concentrated to a minimal volume (~ 1 ml).
			Faecal (human)	Method of Smith & Smith (1989)	Faecal samples were diluted either 1:10 or 1:20 with distilled water and passed through a series of sieves (pore size 50 μm) under negative pressure. The filtered faecal suspension was layered onto a cold discontinuous sucrose gradient consisting of 10 ml of 1.02 g/ml, 25 ml of 1.06 g/ml, and 10 ml of 1.18 g/ml sucrose solutions. After centrifugation (400g/10 min), the middle portion at 1.06 g/ml density was collected and diluted 4 times with distilled water. Cysts were then centrifuged (400g/10 min) and washed 3 times with distilled water.
Kane et al. (1991)	Experimental	USA	Cysts produced *in vitro*	Hypotonic lysis	To isolate cysts, trophozoites were removed by hypotonic lysis in distilled water for 1–24 h at 4ºC, followed by successive washing in distilled water to remove debris.
Sauch et al. (1991)			Faecal (mongolian gerbils)	Sucrose flotation	Flotation of the faecal slurry over 1.0 M sucrose.
				Velocity sedimentation	Crude isolates obtained via sucrose flotation were layered onto a Percoll density gradient ranging from 1.01 to 1.03 g/ml. The sample band was allowed to sediment at room temperature for 1.5 h. After microscopic examination, uncontaminated fractions were collected and washed 3 times with distilled water by centrifugation (650g/2min) to remove Percoll. The cysts were stored at 4°C in distilled water.
Sirlpanth et al. (1995)		Thailand	Faecal (human) Rectal swabs (dogs)	Filtration	Each specimen was emulsified in 0.85% saline solution and filtered through gauze to eliminate large particles. Ten volumes of distilled water were added to the filtrate, which was then centrifuged (400g/10min/room temperature). The sediment containing the cysts was rewashed twice.
Isaac-Renton et al. (1992)		Canada	Faecal (human and animal)	Method of Roberts-Thomson et al. (1976)	A faecal suspension sample was layered on 2.5 ml of 1 M sucrose (density 1.11 g/ml) and centrifuged (400*g*/15min/20°C). Cysts concentrated at the water-sucrose interface were collected, washed by resuspending in 4 ml of normal saline, and sedimented by centrifugation (600*g*/10 min). After removing the supernatant, the cysts were resuspended in normal saline and counted using a hemacytometer.
Uda-Shimoda et al. (2014)		Brazil	Faecal (human)		

Citation	Study type	Country	Sample	Method	Description
Bielec et al. (1996)	Experimental	Canada	Reference isolate (ATCC 30957) Water (recreational and tap)	Percoll–Sucrose gradient by ICR protozoan method	After resuspending the pellet to a packed volume of up to 0.5 ml, it was vortexed with eluting solution to reach a final volume of 20 ml. The vortexed suspension was then layered with 30 ml of Percoll–sucrose flotation solution (density 1.11 g/ml) and centrifuged (1050g/10min). Following centrifugation, the top 20 ml particulate suspension layer, the interface, and 5 ml of the Percoll–sucrose below the interface were transferred. Eluting solution was added to reach a final volume of 50 ml, and the mixture was centrifuged again (1050g/10min). The resulting pellet was resuspended by vortexing.
Hsu & Huang (2000)		Taiwan	Cysts (Waterborne) Water (raw and treated from treatment plant)		
Ahmad et al. (1997)		Malaysia	Water (raw and treated from treatment plant)	Modified method of UK Standing Committee of Analysts	Samples were initially filtered through a polypropylene cartridge filter (pore size 1 μm). The filters were cut and teased apart, and each half of the filter matrix was placed into double–layered plastic bags. These were subjected to stomaching for 10 min in 1 l of 0.1% Tween 80 in distilled water. After allowing the mixture to settle for 24 h at 4ºC, the supernatant was centrifuged (1500g/10min). Subsequently, the samples underwent sucrose flotation using a cold sucrose solution (density 1.18 g/ml), followed by centrifugation (1000g/5min) and washing with PBS.

Citation	Study type	Country	Sample	Method	Description
Hsu & Huang (2000)	Experimental	Taiwan	Cysts (Waterborne) Water (raw and treated from treatment plant)	Immunomagnetic Separation (IMS) by US EPA method 1623	The water samples underwent centrifugation (1500g/10min). IMS was conducted using Dynabeads GC-Combo kit. From the resulting pellet, 0.5 ml was transferred to a tube prefilled with 1 ml of 10 × solution buffer A and 1 ml of 10 × solution buffer B, and distilled water was added to bring the volume to 10 ml. Subsequently, 100 µl of anti-Giardia beads were added, and the sample was rotated for 1 h at room temperature. The tube was placed in a magnetic particle concentrator and gently rocked for 2 min to separate the beads. After removing the supernatant, 1 ml of 1 × solution buffer A was added to each tube. The suspension was transferred into 1.5 ml Eppendorf tubes and subjected to another round of magnetic separation for 1 min. After removing the supernatant and magnet, 50 µl of 0.1 N HCl was added, and the tube was vortexed for 15 s. Tubes were then left to stand vertically for 10 min. The magnet was reinserted to collect the beads, the suspension containing released cysts was transferred to the next tube containing 5 µl of 1 N NaOH.
Castro-Hermida et al. (2008)		Spain	Water (untreated and treated from treatment plant)		
Neto et al. (2010)		Brazil	Water (river)		
Sroka et al. (2013)		Poland	Wastewater (treated)		
Pinto et al. (2016)		Brazil	Cysts (EasySeed)		
de Araújo et al. (2018)			Water (raw from drinking water treatment plants)		

Citation	Study type	Country	Sample	Method	Description
Roubin et al. (2002)	Experimental	France	Cysts (Waterborne) Water (river and for human consumption)	Modified cartridge filtration by AFNOR NF T 90-455	The water samples were filtered using Envirocheck cartridges (pore size of 1 μm). Filtration involved mechanical shaking at 600 rpm for 10 min, which was repeated twice. During the second agitation phase, the cartridge was positioned at a 45° angle.
				IMS by AFNOR NF T 90-455	The volume of the concentrated packed pellet was estimated using a calibrated reference and adjusted to 3, 6, or 10 ml based on the IMS kit validation results. Cyst capture occurred over 60–90 min at room temperature using a rotating mixer. The beads were then collected using a magnet in a magnetic particle concentrator during a 2 min shaking procedure. After discarding the supernatant, the isolated beads were resuspended in 1 ml of buffer and transferred to a microcentrifuge tube placed in a magnetic particle concentrator. Following 1 min of rotation at a 90° inclination, the supernatant was removed. Depending on the type of water analysed, the beads were either rinsed with 1 ml of buffer (for raw waters and certain treated waters) or not (for other water types). The antibody–bead complex was dissociated with 50 μl of 0.1 M HCl, vortexed twice for 10 sec with a 10 min rest at room temperature. This HCl volume was then transferred to 5 μl of 1 M NaOH and homogenized. Raw waters were further diluted with 55 μl of distilled water.
				IMS by Chemunex	The procedure was identical to the one cited above, with the sole modification being the doubling of reagents volumes. The antibody-bead complex was dissociated with 100 μl of 0.1 M HCl, vortexed twice for 10 sec with a 10 min rest at room temperature, then transferred to 10 μl of 1 M NaOH and homogenized.
Cruz et al. (2003)		Portugal	Faecal (human)	Method of Hautus et al. (1988)	Stools were initially broken up in tap water and filtered through a 300 μm mesh sieve. A 3 ml portion of the resulting faecal suspension was layered onto 3 ml of 0.85 M sucrose and centrifuged (600g/10 min/4°C). Cysts located at the sucrose-water interface were aspirated and washed with water. The washed cysts were then layered onto a discontinuous density gradient consisting of two layers: 0.85 and 0.4 M sucrose. After centrifugation (600g/10min/4°C), cysts concentrated at the 0.85-0.4 M sucrose interface were collected and subjected to another wash. Purified cysts were resuspended in distilled water and stored at 4°C.

Citation	Study type	Country	Sample	Method	Description
Ferrari & Veal (2003)	Experimental	Australia	Cysts (Waterborne) Water (backwash)	Filtration	Water concentrates were centrifuged (13000g/10min) and the supernatant was discarded. Samples were resuspended to the initial volume in mAb buffer (2 mM of tetrasodium pyrophosphate, 2% (w/v) bovine serum albumin, and 0.05% (v/v) Tween 80, with pH=8). Samples were filtered (38 μm stainless steel mesh filter) in a Swinnex filter unit with a syringe.
				IMS	A commercial kit (AusFlow IMS kit) specific for *G. lamblia* was obtained from Macquarie Research Ltd. IMS was conducted as described by the manufacturer.
Jaime Massanet-Nicolau (2003)		UK	Sewage (sludge and anaerobically digested) Bovine manure Cysts (University of Arizona)	Sedimentation	A volume of 25 ml of 0.1 M PBS was added to the spiked biosolid sample and vortexed for 60 sec. An additional 25 ml of PBS was added, and the sample was left to stand for 60 min at room temperature. The supernatant was collected and centrifuged (1050g/5min).
				Sucrose flotation	A volume of 10 ml of 0.01% Tween 20 was added to the spiked biosolid sample, and vortexed for 60 sec. A solution of sucrose (density 1.18 g/ml) was injected underneath the sample, followed by centrifugation (1050g/10min). The top 10 ml of the sample, the interface between the layers, and the top 10 ml of sucrose were collected and centrifuged (1050g/10min).
				IMS	The sample underwent purification using a commercial kit for the IMS of *G. lamblia* cysts (Dynal). Initially, paramagnetic beads coated with antibodies specific to *G. lamblia* were incubated with the samples. Subsequently, the cyst-bead complexes were isolated from the sample using magnets and subjected to a double washing process. The cysts were dissociated from the beads, and the beads were removed from the purified sample using magnets.

Citation	Study type	Country	Sample	Method	Description
Bertrand et al. (2004)	Experimental	France	Cysts (Waterborne) Wastewater (raw)	Combination of formalin–ethyl acetate and Percoll-sucrose flotation	A 4-ml pellet of sewage sample was resuspended in 6 ml formalin 10% and 3 ml ethyl acetate, followed by vortexing. Centrifugation (500g/5min) resulted in the formation of 4 layers, with the top 3 being decanted. The resulting pellet was washed with deionized water, centrifuged again and used for Percoll-sucrose flotation. It was resuspended in 20 ml of deionized water and layered onto 30 ml Percoll-sucrose solution (density 1.10 g/ml). After centrifugation (1050g/10min), the aqueous suspension and 5 ml Percoll-sucrose solution under the interface were collected. The collected material was washed with an equal volume of deionized water by centrifugation (2000g/10min). Two additional washings were carried out, and the pellet was conserved in deionized water at -80ºC.
Bertrand et al. (2009)		Luxembourg	Cysts (Waterborne) Faecal (human and dog) Wastewater (abattoir and treatment plant)		
Polverino et al. (2004)		Argentina	Faecal (human and dog)	Centrifugation	1. Suspension of faecal matter in Telemann solution (50 ml of formalin 40% and 5g of NaCl in 950 ml of distilled water) with 2 ml of ether. Followed by centrifugation (1500rpm/5min). The supernatant was discarded, and the pellet was washed with PBS (pH 7.2).
				Sucrose flotation	2. Suspension of faecal matter in PBS, followed by centrifugation (1500rpm/5min). PBS was added and resuspended by vortex. Then, 4 ml of sucrose solution (density 1.275 g/ml) was added and centrifuged (1500 rpm/5min). The sucrose phase and the pellet were transferred, and 2 volumes of PBS were added. The cysts were concentrated by centrifugation (2000rpm/5min). The sediment was resuspended with PBS in a final volume of 0.5 ml and stored at 4 °C.
				Combination of centrifugation and sucrose flotation	3. Application of 1 plus 2 on the resulting pellet.

Citation	Study type	Country	Sample	Method	Description
Santos et al. (2004)	Experimental	Brazil	Water (sludge)	Filtration	Filtration with a 1 mm^2 plastic sieve to remove large debris.
				Modified method of Robertson et al. (2000)	Combination of ether clarification procedure (ECP) and sucrose flotation method (SFM). For ECP, aliquots of filtrate were diluted (1:3) with 1% Tween 80 solution. The pellets were retrieved by double centrifugation (1500g/15min), followed by further concentration using ECP (1500g/10min), after manual agitation (30 sec). For SFM, following double centrifugation, a saturated sucrose solution (1.20 g/ml) was added to the pellets and centrifuged again (1500g/15min). The superficial layer (3 ml) was collected and transferred, and this procedure was repeated.
Alvarado & Wasserman (2006)		Colombia	Faecal (human)	Method of Alvarado & Wasserman (2006)	The samples were diluted with distilled water and filtered through gauze to remove coarse material. The filtrate was then centrifuged (800g/5min), and the supernatant was discarded. The resulting pellet was washed, resuspended in distilled water, and divided into four aliquots of 5 ml. Each aliquot was placed over 3 ml of cold 0.85 M sucrose and centrifuged (600g/10min). The interfaces were recovered, mixed, and diluted 25-fold with distilled water. The diluted mixture was vacuum filtered through a 5 µm cellulose acetate membrane. The filter was washed, and the cysts collected on the membrane were sedimented by centrifugation (800g/5min). The purified cysts were stored at 4°C with antibiotics (1000 U penicillin and1 mg/ml streptomycin).
Ferrari et al. (2006)		Australia	Cysts (Waterborne) Wastewater (abattoir)	Combination of calcium carbonate flocculation and filtration	Addition of 10 ml of 1 mol $CaCl_2$ and 10 ml of 1 mol $NaHCO_3$ to the water samples (pH 10.0). The samples were centrifuged and washed with a buffer solution and 0.05% Tween 80 solution. The samples were prefiltered through a series of 50 and 38 µm stainless steel mesh filters.
				Combination of immunofluorescence and flow cytometry	The cyst wall-specific IgG1 mAb G203, conjugated to fluorescein isothiocyanate, were used for sample prestaining at concentrations of 2 mg/ml and 4 mg/ml, respectively, in buffer. The samples were vortexed and incubated in the dark at 4°C for 15 min. Fluorescence activated cell sorting (FACS) was performed on a BD FACSCalibur-Sort equipped with a 488 nm argon ion laser for excitation. The FACSCalibur was equipped with a SortStage attachment, which allowed for the capture of sorted cells onto 13 mm (pore size 0.8 µm) isopore polycarbonate membranes.

Citation	Study type	Country	Sample	Method	Description
Karanis et al. (2006)	Experimental	Bulgaria	Water (Russia: drinking water and river; Bulgaria: river, lake, well, tap, spring, sewage and bottled)	Combination of filtration and sucrose flotation	The water samples underwent filtration using a polypropylene cartridge filter with 1 mm porosity. The filters were cut open and washed twice in 0.1% Tween 80 solution, followed by centrifugation (2100g/10min/4ºC). These processes were repeated twice. The resulting pellets were layered over an equal volume of sucrose 2.5 M and centrifuged (300g/13min/4ºC). The supernatants were carefully transferred and washed twice with distilled water (2100g/10 min/4ºC). Further purification of the samples, using 1.5 M sucrose, was performed when necessary.
				Aluminum sulfate flocculation	An aqueous aluminum sulfate solution (16 mg Al^{3+}/l) was added to each water sample, with pH adjusted to 5.4–5.8. The samples were left overnight in the dark at room temperature to allow flocculation and precipitation. The resulting pellet was centrifuged (2100g/10 min/ 4ºC) and then resuspended with a lysis buffer (8.4 g citric acid monohydrate; 17.64 g trisodium citrate dihydrate; distilled water up to 100 ml; pH 4.7). After settling with the lysis buffer for 1 h at room temperature, the pellet underwent a double washing with distilled water.
				Discontinuous sucrose gradient	Sheather's sugar solution (500 g sucrose; 6.5 g phenol; 320 ml de-mineralized water) was diluted with 0.1 M PBS (pH 7.2) to solutions A (Sheather/PBS 1:2) and B (Seather/PBS 1:4), supplemented with a few drops of 1% Tween 80. A portion of 15 ml of the solution B was layered over 15 ml of solution A. Then, the 10 ml sample suspensions were laid over solution B, and the gradients were centrifuged (1200g/30min/4ºC). The resulting supernatants were transferred and washed twice with distilled water. The final pellets were transferred to Eppendorf tubes and stored at 4ºC until use.
Neto et al. (2010)		Brazil	Water (river)	Calcium carbonate flocculation	A solution of $CaCl_2$ and a solution of $NaHCO_3$ were added separately to the sample (pH 10.0). Samples were kept overnight at room temperature. The supernatant was aspirated, and the precipitate was dissolved with 10% sulfamic acid. The suspension was centrifuged, and the supernatant was aspirated. The pellet material was centrifuged again.
				Filtration	The samples were filtered through mixed cellulose esters membranes (with 3 mm porosity). Membranes were scraped and manually rinsed with eluting solution (0.1% Tween 80). The resulting liquid was centrifuged, and the pellet rinsed with Milli-Q® water.

Citation	Study type	Country	Sample	Method	Description
Ramadan et al. (2010)	Experimental	Singapore	Water (tap, secondary effluent and purified Milli-Q®) Cysts (ColorSeed)	IMS with magnetic tube holder	The samples were incubated for 1 h with anti-body-coated magnetic beads (Dynabeads® GC-Combo kit). The magnetic-labelled protozoan cells were separated from the liquid matrix using a magnetic particle concentrator. The supernatant was decanted to recover any remaining magnetic particles.
				IMS with Magnetic Separation System (MSS)	The samples were incubated for 1 h with anti-body-coated magnetic beads (Dynabeads® GC-Combo kit). The magnetic-labelled protozoan cells were separated from the liquid matrix using the MSS. The sample was injected, and the glass flat-sided tube was rinsed four times with filtered PBS. After each rinse, the liquid solution was injected. Once the entire sample was injected, filtered PBS was used to rinse the fluidic system, trapping the magnetic labelled cells and beads in the separation chamber. The supernatant from the first magnetic separation was processed through the MSS again.
Barazesh et al. (2011)		Iran	Faecal (human)	One-phase sucrose gradient	The sediments were dissolved in a 0.5% Tween 80 solution and centrifuged (700g/5min). This process was repeated until the supernatant was clear. The sediments were then collected, prepared into suspensions with appropriate concentrations by adding distilled water and were decanted into tubes containing equal volumes of 0.85 M sucrose solutions. The tubes were centrifuged (500g/20min). The thin layer formed between the distilled water and sucrose phases was centrifuged 2-3 times (500g/5min) to remove the sucrose. The resulting sediments were then dissolved in distilled water to create suspensions.
				Two-phase sucrose gradient	The sediments were dissolved in 0.2 M PBS and centrifuged (500g/5min), with repeated washing until the supernatant was clear. The sediments were then dissolved in distilled water, poured into tubes containing equal volumes of 1.5 M sucrose solution and centrifuged (1700g/10min). The contents of the intermediate phases were then centrifuged again (300g/5min/4°C) to remove sucrose. The obtained sediments were dissolved in distilled water and added to a 0.75 M sucrose solution. The mixture was centrifuged (1700g/10min), allowing the cysts to settle while cellulose particles aggregated at the intermediate phase.

Citation	Study type	Country	Sample	Method	Description
Barazesh et al. (2011)	Experimental	Iran	Faecal (human)	Percoll-sucrose gradient	The sediments were dissolved in distilled water and centrifuged (500g/5min), with repeated washing until the supernatant was clear. The sediments were then dissolved in distilled water and added to equal volumes of 1 M sucrose solution, followed by centrifugation (500g/10min). The middle phase contents were collected and washed 2-3 times with distilled water. The suspension was then added to two Percoll solutions (densities of 1.05 and 1.09 mg/ml) and centrifuged (500g/20min). The contents of the phase created between the two Percoll layers were collected, washed 2-3 times, and mixed with distilled water.
				Modified two-phase method	The sediments were dissolved in 0.5% Tween 80 and centrifuged (500g/5min), with repeated washing until the supernatant was clear. The sediments were then dissolved in distilled water and layered onto an equal volume of 1.5 M sucrose, followed by centrifugation (1300g/10 min/4°C). The intermediate phase contents were washed 2-3 times by centrifugation (500g/5 min), then dissolved in distilled water. The solution was added to 0.85 M sucrose and centrifuged (1600g/10min/4°C).
Keserue et al. (2011)		Switzerland	Cysts (Waterborne) Water (tap, pond and river) Wastewater	IMS	A 5 ml sample was passed through the MACS® MS column at a flow rate of 2.05 ml/min using a peristaltic pump. The column was then washed twice with 3 ml of PBS, twice with 3 ml of PBST (PBS with 0.01% Tween 20), and once with 3 ml of PBS at a flow rate of 2.39 ml/min. After removing the column from the magnet, purified cells were recovered by flushing 1 ml of PBS through the column into a 15-ml tube using the provided plunger.
Zhang et al. (2013)		China	Cysts (Waterborne) Water (tap, reservoir, river and sewage) Wastewater (treatment plant)	Coagulation collection	Each sample underwent rapid mixing at 180 rpm for 2 min, followed by slow mixing at 40 rpm for 15 min, and then settling for 30 min. The coagulant (polyaluminium chloride) was added during the rapid mixing period. During coagulation, metal ions hydrolysed into polymeric substances and adsorbed colloidal particles, forming visible flocs which entrapped the cysts. The supernatant was discarded, leaving approximately 15 ml of flocs/l. The pH of the metal hydroxide solution was then adjusted to 2.0, triggering an acid-base neutralization reaction that dissolved the flocs and released the cysts. The samples containing the cysts were filtered through membranes (pore size 0.45 µm). The cysts were trapped on the membrane and rinsed with PBS at pH 3.0 and pH 8.0.

Citation	Study type	Country	Sample	Method	Description
Uda-Shimoda et al. (2014)	Experimental	Brazil	Faecal (human)	Zinc sulfate flotation	After filtering a homogeneous mixture of faeces and water, the strained faecal suspension undergoes centrifugation, and the sediment is then resuspended in a zinc sulfate solution (density 1.18 g/ml). The suspension undergoes centrifugation again, which results in the flotation of the cysts.
				Sedimentation in water	A homogeneous mixture of faeces and water was filtered, and then left to sediment for 12 h. The resulting sediment was then resuspended in distilled water and allowed to settle again. The washing step was repeated multiple times until the sample was considered cleaned.
El-Adaway et al. (2017)	Cross sectional	Cairo		Sucrose density-gradient	A stool sample was initially diluted with distilled water and filtered through gauze to remove the coarse material. The filtrate was centrifuged (800g/5min), and the supernatant was discarded. The resulting pellet was vortexed and divided into two equal portions. Only the second portion underwent the sucrose density-gradient method. These stool samples were diluted 1:10 in distilled water, vortexed, and 5 ml of the resulting stool pellet was layered onto 10 ml of 1 M sucrose solution. The mixture was centrifuged (450g/5min), and the resulting pellet was subjected to another centrifugation under the same conditions. The final pellet was resuspended in 2.5ml of distilled water and layered onto 10 ml of 0.5 M sucrose solution, followed by centrifugation (450g/5min). The bottom 1 ml of liquid was collected from which 200µl were dispensed into tubes and stored at 20°C.
Dehghani-Samani et al. (2019)	Experimental	Iran		Sucrose-water floatation	A stool sample was diluted in 100 ml tap water and filtered through a 300 µm filter. A 3 ml aliquot of the filtered faecal suspension was mixed with 3 ml of 0.85 M sucrose and centrifuged (2000–3000rpm/10min). The cysts at the sucrose-water interface were aspirated and washed 3 times with water. The purified cysts were layered onto a discontinuous density gradient consisting of 3 ml layers of 0.85 M sucrose. After centrifugation (2000–3000rpm/10min), cysts concentrated at the sucrose interface were collected and washed again.

Citation	Study type	Country	Sample	Method	Description
Terrones et al. (2019)	Observational	Peru	Faecal (human)	Spontaneous sedimentation in tube	Water or other low-density liquids, such as physiological saline solution, are used to recover the evolutionary microscopic forms of the parasites. These forms settle at the bottom of the tube, deposited there due to their density.
				Faust method	A solution of zinc sulfate (density 1.18 g/ml) was used, causing the residue to settle at the bottom of the tube. The supernatant was then centrifuged (2300 rpm), followed by 3 washes with distilled water to remove zinc sulfate.
				Single-phase sucrose gradient	A saline solution was added to the pellet and centrifuged (1500rpm/5min). Subsequently, 4 ml of PBS and 4 ml of sucrose solution (density 1.275 g/ml) were added. The mixture was then centrifuged (2300rpm/10min). The sucrose sediment phase was aspirated, followed by 3 washes with distilled water to remove sucrose.
				Two-phase sucrose gradient	The pellet was mixed with 0.85 M sucrose solution, followed by centrifugation (2300rpm/10min/4°C). The sucrose-water phase was collected, and the pellet underwent 3 washes with distilled water. A gradient tube ranging from 0.85 M to 0.4 M sucrose was prepared. The pellet was added, then centrifuged (2300rpm/10min/4°C). The interface was collected, followed by another centrifugation (2300rpm/5min/ 4°C) to remove sucrose.
Bezagio et al. (2020)	Experimental	Brazil		Modified Ritchie method	One gram of faeces was diluted in 0.85% saline solution, then filtered through gauze and centrifuged (1200g/5min). Several types of water (Milli-Q® water, distilled water, deionized water and injection water) were added along with ethyl ether (6:4 ml). After vigorous stirring, the samples were centrifuged (1200g/2min). The final sediment received the amount of each respective reagent necessary to complete 150 µl. A portion of 50 µl was used for quantification in a haemocytometer.

Citation	Study type	Country	Sample	Method	Description
Lyu & Wen (2020)	Experimental	China	Intestines of host (animal)	Method of Lyu & Wen (2020)	The intestines of the host animals were dissected into segments and transferred into TYI-S-33 medium, where they were chilled on ice for over 30 min. The suspension was centrifuged (1000g/1s), and the supernatant was transferred to a new tube, which was then centrifuged (750g/5min). After discarding the supernatant, the sediment was resuspended with TYI-S-33 and kept at 37°C for 30 min. The TYI-S-33 was replaced with fresh TYI-S-33 and incubated at 37°C for another 30 min, repeating this step once more. Subsequently, the tube was chilled on ice for 30 min, centrifuged (2000g/5min), and the supernatant was discarded. The trophozoites were collected from the pellet and resuspended in PBS.
			Faecal (animal)		Faeces samples were suspended in water and filtered through a 60-mesh and a 200-mesh sieves. The resulting filtrate was centrifuged (1500g/10min) and the supernatant was discarded. To the sediment, a 33% zinc sulfate solution of equal volume was added for resuspension, followed by centrifugation (1200g/15min). The supernatant was transferred and diluted with 4 volumes of water before another round of centrifugation (500g/10min). The last four steps were repeated twice to purify the cysts, and the precipitated cysts were resuspended with PBS.

Caption: $CaCl_2$ – Calcium chloride; CDW – Cold Distilled Water; ECP – Ether Clarification Procedure; FACS – Fluorescence Activated Cell Sorting; HBSS – Hank's Balanced Salt Solution; HCl – Hydrogen chloride; IMS – Immunomagnetic Separation; MSS – Magnetic Separation System; NaCl – Sodium chloride; $NaHCO_3$ – Sodium bicarbonate; NaOH – Sodium hydroxide; PBS – Phosphate-buffered saline; PBST – PBS with 0.01% Tween 20; SFM – Sucrose Flotation Method; UK – United Kingdom; USA – United States of America.

2.4.3. Methods for the isolation and purification of *G. lamblia*

A total of 56 methods were identified for the isolation and purification of *G. lamblia* (Table 4). The initial separation of the organism from the rest of the sample, which often contains large amounts of debris and contaminants, was primarily achieved through a combination of filtration, low-speed centrifugation, and multiple washing steps with solutions such as Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), or distilled water. For further purification of the sample, the two most cited methods were density gradients and immunomagnetic separation (IMS).

Density gradient centrifugation or flotation with sucrose or Percoll® solutions, with densities ranging between 1.10 and 1.275 g/ml, was commonly employed for faecal samples. The principle of faecal flotation is based on a gradient produced by the density difference between the solution used and the target sample (parasitic elements). Sucrose or Percoll® solutions are denser than parasitic elements, enabling their isolation at the surface (Ballweber et al., 2014). Polverino et al. (2004) compared three techniques for the isolation and purification of *G. lamblia* cysts and concluded that techniques 2 (sucrose flotation) and 3 (a combination of centrifugation and sucrose flotation) left the least amount of debris in the purified cyst suspension. Terrones et al. (2019) compared four techniques for the isolation and purification of *G. lamblia* cysts and concluded that a two-phase sucrose gradient was most effective. Factors such as the quantity of examined material, dilution factor used, whether centrifugation is performed, the length of time allowed for flotation, and the type and specific gravity of the flotation solution used can influence the ability to detect parasites and the yield of the flotation process (Ballweber et al., 2014).

IMS was also quite common in the cited studies, particularly when the sample being analysed was from a water source or when it was intended for further deoxyribonucleic acid (DNA) extraction. The IMS purification step involves the selective separation of *Giardia spp.* cysts with magnetic microspheres covered with purified antibodies against the cysts. The bead–organism complex undergoes a dissociation step, usually by either acid or heat dissociation (Ogura & Sabogal-Paz, 2021; Pinto et al., 2016). Neto et al. (2010) compared calcium carbonate flocculation and membrane filtration, followed or not by an IMS step, and concluded that higher concentrations of cysts were detected when IMS was employed for sample purification. Pinto et al. (2016) compared two dissociation procedures (acid and heat) in the IMS method and

concluded that acid dissociation was more efficient for *Giardia spp.* cysts. Ferrari & Veal (2003) showed that IMS produces significantly higher recovery rates in samples with low turbidity, but the efficiency of IMS decreases as turbidity increases. Hsu & Huang (2000) compared two purification methods, the Percoll®–sucrose density gradient (ICR protozoan method) and IMS (Method 1623) and found that the average recovery efficiency for *Giardia spp.* with Method 1623 was 48.0% greater than that with the ICR protozoan method. Despite its higher cyst recovery efficiency, the cost of Method 1623 poses a challenge for its application in developing countries (Hsu & Huang, 2000). Several factors, such as sediment volume, sample volume and turbidity, magnetic material concentration, antibody type, reagents and conditions used, and pH, may affect the efficiency of the IMS method (Ogura & Sabogal-Paz, 2021; Pinto et al., 2016; Roubin et al., 2002). Sucrose gradient flotation can also be combined with IMS, as it allows for detailed analysis of the *G. lamblia* genome in clinical stool samples (Hanevik et al., 2015).

2.4.4. Methods for the excystation of *G. lamblia*

For the excystation of *G. lamblia*, 7 methods were identified, as presented in Table 5. In the reviewed studies, excystation was generally performed in two phases: an initial low-pH induction phase using acid solutions (pH≤2.0) and a subsequent excystment phase. The first phase was employed in nearly all cited studies and comprised incubating cysts with aqueous hydrogen chloride (HCl) or a pepsin–acid solution for periods of 5–120 min at 37°C. The second phase exhibited variation in the cited methods. Some protocols use enzymes such as trypsin dissolved in Tyrode solution or cysteine HCl/ascorbic acid mixtures in HBSS to facilitate excystment, while others neutralize the acid solution with sodium bicarbonate before resuspending the cysts in fresh culture medium (Cruz et al., 2003; Douglas et al., 1987; Isaac-Renton et al., 1992; Kane et al., 1991). Moreover, the excystment phase often involves specific incubation conditions, such as maintaining the cysts in an inverted position at 37°C and using various growth media (TYI-S-33 or HSP-3) with a pH ranging from 7.0 to 7.8 (Alvarado & Wasserman, 2006; Douglas et al., 1987). The conditions for *in vitro* excystation of *G. lamblia* mimic the natural process that occurs within the digestive system of an infected host. Gastric acid (pH 1.5–2.5) initiates excystation by creating an acidic environment similar to laboratory-induced conditions (pH≤2.0). Upon passage through the stomach, cysts encounter the neutral pH environment of the small intestine (pH 7.0–8.0), similar to the solutions used *in vitro* to neutralize acid-induced excystation (Schaefer et al., 1984). Furthermore, Isaac-Renton et al.

(1992) performed *in vivo* excystation by inoculating cysts into gerbils, followed by examination of the small intestinal contents of trophozoites after a specified period, providing insights into the excystation process under real physiological conditions.

Table 5 – Methods for excystation of *G. lamblia*.

Citation	Study type	Country	Sample	Method	Description
Douglas et al. (1987)	Experimental	USA	Faecal (human)	Method of Rice & Schaefer (1981)	Excystation medium (pH 8.0): 0.5% (wt/vol) trypsin (1:100) dissolved in Tyrode solution. Low-pH induction involved incubating a cyst suspension with a mixture of 5 ml of aqueous HCl (pH 2.0); 2.5 ml of HBSS (supplemented with 29 mM L-cysteine hydrochloride and 67 mM glutathione); and 2.5 ml of 0.1 M $NaHCO_3$for 30 min at 37°C. After centrifugation (1000g/2min), the cysts were resuspended in excystation medium and incubated in an inverted position at 37°C.
Al-Tukhi et al. (1991)		Saudi Arabia		Modified method of Bingham & Meyer (1979)	A cyst suspension was mixed with aqueous HCl (1:10, pH 2.0) and incubated upright at 37°C for 1 h. After centrifugation (600g/10min), the pellet was collected, inoculated into prewarmed modified TYI-S-33 medium and incubated overnight in an inclined position to allow complete hatching.
Alvarado & Wasserman (2006)		Colombia			Excystation medium: aqueous HCl (pH 2.0). HSP-3 medium (pH 7.0): 85 ml Hanks-phytone broth; 20 ml filter-sterilized heat-inactivated human serum; 7.5 ml NCTC-135; 1.5 ml 1.0 M $NaHCO_3$; 200.000 U potassium penicillin G; 0.01 g streptomycin sulfate and 0.01 g gentamicin sulfate. A cyst suspension was inoculated in excystation medium (1:10) and incubated at 37 °C for 1 h. Following centrifugation (600g/5min), the pellet was washed with water, recentrifuged, and then resuspended in 0.5 ml of HSP-3 at 37 °C. The suspension was placed in a depression slide chamber, incubated inverted at 37 °C for 1 h, and subsequently examined at 300x magnification for parasite counting.
Bertrand et al. (2009)		Luxembourg	Cysts (Waterborne) Faecal (human and dog) Wastewater (abattoir and treatment plant)		Excystation medium (pH 2.0): pepsin-acid solution with 25 mM $NaHCO_3$, 12 mM KCl, 40 mM NaCl, 6 mM $CaCl_2$, 1500 U/ml pepsin. A portion of the cyst suspension was added to a 1 ml of pepsin-acid solution and incubated at 37ºC for 1 h. The suspension was then centrifuged (800g/3min), and the resulting pellet was washed twice with PBS and resuspended in TYI-S-33 at 37ºC.

Citation	Study type	Country	Sample	Method	Description
Kane et al. (1991)	Experimental	USA	Cysts produced *in vitro*	Combination of the methods of Boucher & Gillin (1990), Buchel et al. (1987), Schupp et al. (1988)	Excystation medium: 12 mM cysteine HCl/ascorbic acid in HBSS containing 1500 U pepsin/ml. Cysts were incubated with excystation medium at 37ºC for 30 min. The medium was then neutralized by the adding 1 N $NaHCO_3$and centrifuged (1000g/5min). The resulting pellet was resuspended in TYI-S-33 and incubated at 37ºC.
					The cysts exposed to the cysteine/ascorbic acid solution (with or without pepsin) were subsequently incubated for 30 min at 37ºC with 1 mg/ml α-chrymotrypsin I-S in Tyrode's solution (pH 8.0). The excystation medium was then replaced with TYI-S-33, and tubes were incubated at 37ºC and periodically examined for the presence of trophozoites.
Isaac-Renton et al. (1992)		Canada	Faecal (human and animal)	Method of Sauch (1987)	Excystation medium: HCl-saline (concentrated HCl and 0.85% NaCl, pH 1.4), HBSS containing 32 mM glutathione with 57 mM L-cysteine, and 0.1 M $NaHCO_3$. The excystation medium was added to the cyst suspension and incubated for 45 min at 37°C. After centrifugation (650g/2min), the pellet was resuspended in Tyrode salt solution. An aliquot was removed and examined microscopically, while the remainder of the suspension was inoculated into prewarmed modified TYI-S-33 medium containing 100 mg of L-cysteine in HCl and 100 mg of dried bovine bile per 100 ml.
			Mongolian gerbils	Method of Belosevic et al. (1983)	A random animal was sacrificed, and an aliquot of the small intestinal contents was examined for trophozoites. None-infected gerbils were gavage-fed 20 mg of metronidazole daily for 3 days and given dexamethasone (20 mg in 5 ml of PBS). A suspension of cysts in distilled water was inoculated into a gerbil, and 5-7 days later, the gerbil was sacrificed. The small intestine was removed, irrigated with prewarmed TYI-S-33, and the mucosa was scraped. The collected material was pooled and examined microscopically.

Citation	Study type	Country	Sample	Method	Description
Sirlpanth et al. (1995)	Experimental	Thailand	Faecal (human) Rectal swabs (dogs)	Method of Sirlpanth et al. (1995)	Excystation medium: 0.4 pepsin in HCl, pH 1.65 TYI-S-33 medium: containing 10 ml of heat-inactivated human serum, 2.5 ml of NCTC-135, 1.0 mg of piperacillin and 1.0 mg of amikacin per 100 ml. Cysts were inoculated into excystation medium and incubated at 37°C for 1-2 h. After centrifugation (200g/10min), the cysts were washed twice with distilled water and incubated with TYI-S-33 at 37°C in an inclined position. The medium was changed daily for 3 days and subcultures were performed when the pH of the culture medium dropped below 6.4.
Cruz et al. (2003)		Portugal	Faecal (human)	Method of Schupp et al. (1988)	Excystation medium: dilution of 100 mM cysteine-ascorbic acid solution in Eagle's minimal essential medium solution in HBSS. Cysts were inoculated into culture tubes containing warm excystation medium (37°C) and incubated for 15 min at 37ºC. The cysts were centrifuged, and the resulting pellet was resuspended in TYI-S-33. The tubes were incubated at 37°C and examined microscopically for 48 h.

Caption: $CaCl_2$ – Calcium chloride; HBSS – Hank's Balanced Salt Solution; HCl – Hydrogen chloride; KCl – Potassium chloride; NaCl – Sodium chloride; $NaHCO_3$ – Sodium bicarbonate; NaOH – Sodium hydroxide; PBS – Phosphate-buffered saline; USA – United States of America.

2.4.5. Methods for the axenization of *G. lamblia*

For the axenization of *G. lamblia,* 3 methods were identified, as presented in Table 6. Most cited studies involved incubating trophozoites at 37ºC with Keisters' modified TYI-S-33 medium, using different concentrations and combinations of antimicrobials (such as penicillin, streptomycin and amphotericin B) to prevent bacterial and fungal contamination (Keister, 1983). The culture medium commonly has an alkaline pH ranging from 7.0 to 7.2 and is often supplemented with bile salts and bovine serum.

Table 6 - Methods for the axenization of *G. lamblia* trophozoites.

Citation	Study type	Country	Sample	Method	Description
Gordts et al. (1985)	Experimental	Belgium	Duodenal fluid (human)	Method of Gordts et al. (1985)	Culture medium: TPS-1 medium with trypticase peptone, liver digest, glucose, L-cysteine, ascorbic acid, supplemented with 10% foetal calf serum, 10% NCTC-135 with L-glutamine, and a mix of antibiotics (penicillin (100 IU/ml), streptomycin (100 mg/l), vancomycin (20 mg/l), and clindamycin (20 mg/l)). Duodenal fluid was incubated with culture medium at 37°C and screened for living trophozoites after 2 h. Cultures were inspected daily for 10 days.
Al-Tukhi et al. (1991)		Saudi Arabia	Faecal (human)	Modification of the method of Keister (1983)	After 5 days of excystation, subculturing was done with fresh TYI-S-33 medium. During growth, trophozoites formed a layer, which was dislodged by immersion in ice-water for 15 min and centrifuged (600 g/10min/4°C). The trophozoites were resuspended in 5 ml of fresh medium.
Kane et al. (1991)		USA	Cysts produced *in vitro*		Filter-sterilized TYI-S-33 (pH 7.1) containing 10% bovine serum; 0.5 mg/ml of bovine bile; 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. Trophozoites were maintained axenically by subculturing twice a week and were grown to confluence (usually 66-72 h) at 37ºC.
Isaac-Renton et al. (1992)		Canada	Faecal (human and animal)		Excysted material was inoculated with filter-sterilized TYI-S-33 containing 500 IU/ml Penicillin G, 50 µg/ml streptomycin, and 10 µg/ml amphotericin B.
					After *in vivo* excystation, intestinal washes and scrapings were examined microscopically and trophozoites were counted with a haemocytometer. A diluted trophozoite suspension was inoculated with TYI-S-33 at 37°C.
Cruz et al. (2003)		Portugal	Faecal (human)		Trophozoites were cultured and axenized in modified TYI-S-33.
Bénéré et al. (2007)		Belgium	Reference isolate (ATCC 30957) Laboratory isolate (Swiss Tropic Institute)		Trophozoites were grown in modified TYI-S-33 (pH 7.0), supplemented with 10% heat inactivated foetal calf serum and 0.05% bovine bile. The tubes were filled to 90–95% of total capacity and incubated at 37 °C, with subculturing 3 times a week.

Citation	Study type	Country	Sample	Method	Description
Uda-Shimoda et al. (2014)	Experimental	Brazil	Reference isolate (ATCC 30888)	Modification of the method of Keister (1983)	Trophozoites were maintained under axenic culture in TYI-S-33 medium.
Sirlpanth et al. (1995)		Thailand	Faecal (human)	Method of Sirlpanth et al. (1995)	Axenic cultures were obtained by multiple washes with TYI-S-33 containing decreasing amounts of amphotericin B (10.5, 2 to 1 μg/ml) by centrifugation (200g/10min). The cultures were established once no contamination (fungal – Sabouraud agar; bacterial – MacConkey agar, blood agar, thioglycolate broth) was observed.

2.4.6. Methods for the encystation of *G. lamblia*

For the encystation of *G. lamblia,* 3 methods were identified, as presented in Table 7. In the reviewed studies, encystation was induced using filter-sterilized TYI-S-33 media supplemented with different concentrations of bovine bile, adjusted to pH 7.8. The presence of bovine bile in the medium and an alkaline pH are essential factors for inducing encystation, as both mimic the conditions found in the small intestine that trigger cyst formation *in vivo* (Eckmann et al., 2000; Gillin et al., 1989). In the method used by Bielec et al. (1996), lactic acid was also included in the encystation medium, as this compound has been shown to stimulate encystation (Gillin et al., 1989). The inclusion of antimicrobials in the encystation medium was optional, which may be due to the specific aims of the studies, such as the need to replicate the natural conditions of the small intestine where natural interactions can occur between *G. lamblia* and the gut microbiota.

Table 7 – Methods for the encystation of *G. lamblia.*

Citation	Study type	Country	Sample	Method	Description
Kane et al. (1991)	Experimental	USA	Reference isolates (ATCC 30888 and ATCC 30957) Laboratory strain (LT and WB clones) Cat strain	Method of Kane et al. (1991)	Encystation medium: filter-sterilized TYI-S-33 medium (pH 7.8) containing 10% bovine serum; 10 mg/ml of bovine bile; 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin.
					Confluence trophozoites were incubated with encystation medium at 37ºC for 96 h.
					After 24 h in the encystation medium, cultures were pelleted by centrifugation (500g/10min), resuspended in TYI-S-33 (pH 7.1), and incubated at 37°C. Cysts were harvested by centrifugation (500g/10min) and washed twice in 20 mM sodium phosphate buffer (pH 7.1).
				Production of cysts in roller bottles	Trophozoites were grown to confluence in TYI-S-33 (pH 7.1) within roller bottles at 37°C at 1 rotation/h. The unattached trophozoites and TYI-S-33 were discarded, and encystation medium was added. The roller bottles were incubated at 37°C for 24 h. The bottles were immersed in ice for 30 min and the contents were centrifuged (150g/30min). The encysting trophozoites were resuspended in TYI-S-33 and returned to the roller bottles for an additional 24 h at 37°C. The parasites were harvested by immersion in ice for 30 min and centrifugation (150g/30min). Cysts were washed and incubated in distilled water.
Bielec et al. (1996)		Canada	Reference isolate (ATCC 30957) Water (recreational and tap)	Modified method of Boucher & Gillin (1990)	Cultures were incubated at 37.5ºC for 66 h in encystation medium (TYI-S-33, pH 7.8) with bovine bile (5 mg/ml) and lactic acid (0.6 mg/ml), without antibiotics).

2.5. Conclusion

G. lamblia is one of the most common waterborne parasites that infects humans. The cyst stage of this parasite remains viable in the environment until ingestion by a new host and has been extensively identified in various water sources accessed by humans and animals. The present systematic review identified a range of methods used throughout the *G. lamblia* life cycle, including isolation, purification, axenization, excystation and encystation and highlights the diversity and effectiveness of currently employed methods. Across the reviewed studies, several common techniques emerged with minor modifications. The methods used for the isolation and purification of *G. lamblia* are particularly notable because of their variability. While other stages of the parasite's life cycle have more standardized procedures, the isolation and purification methods still lack uniformity and efficiency. Therefore, it is essential to develop and implement new methods, or optimize existing methods to be faster, less expensive, and easier to perform. Despite considerable progress, ongoing research and standardization are crucial to further improve these methods and to establish best practices.

2.6. References

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3. Chapter 2 – A comprehensive analysis of viability assessment methods for *Giardia lamblia* and *Trichomonas vaginalis* trophozoites: a systematic review

3.1. Abstract

Giardia lamblia and *Trichomonas vaginalis* are flagellated protozoan parasites that often cause asymptomatic infections but may lead to gastrointestinal or genitourinary symptoms. Due to emerging resistance, improved treatment options are needed. However, selecting an appropriate method for assessing the *in vitro* susceptibility of *G. lamblia* and *T. vaginalis* in the presence of potential therapeutic compounds remains challenging due to the variability in these methods. This study aims to provide an overview of commonly employed methods for determining trophozoite viability in the presence of potential therapeutic compounds and to propose a standardized viability assay for susceptibility testing in *G. lamblia* and *T. vaginalis*. A systematic literature review was conducted according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA 2020) statement, using databases including MEDLINE, ScienceDirect, and Web of Science, with the following search equation: "*in vitro* "AND "method" AND ("susceptibility" OR "viability" OR "sensitivity") AND ("giardia" OR "trichomonas"). The search identified 32 experimental studies with diverse viability assays. For *G. lamblia*, the MTT assay, adherence inhibition assay and [3H]-thymidine incorporation assay were prominent. In *T. vaginalis*, the trypan blue assay, motility assessment, and resazurin assay were frequently used. These findings underscore the diversity in viability assessment methods, highlighting the importance of standardizing viability assays to ensure accurate and reproducible results in drug susceptibility studies. The fluorometric resazurin assay has emerged as a suitable choice for standardization in both parasites, offering cost-effectiveness, reliability, and ease of use.

Keywords: cell viability, giardiasis, parasitic diseases, parasite viability, protozoan parasites, trichomoniasis.

3.2. Introduction

Giardia lamblia (syn. *G. intestinalis*, *G. duodenalis*) is a flagellated protozoan parasite responsible for giardiasis, a common intestinal parasitic infection worldwide. The disease is most often asymptomatic, particularly in young adults, and tends to be self-limiting. However, when symptomatic, it manifests with symptoms such as fatigue, abdominal distension, acute diarrhoea, and other chronic gastrointestinal symptoms. The life cycle of *G. lamblia* comprises two distinct morphological forms: trophozoites, which infect the duodenum, and cysts, which are responsible for disease transmission by contaminating water or food sources after excretion in faeces (Leung et al. 2019; European Centre for Disease Prevention and Control (ECDC) 2022). In 2019, 18.004 confirmed giardiasis cases were reported in the European Union and European Economic Area (ECDC 2022).

Trichomonas vaginalis, another flagellated protozoan parasite, is the etiological agent of trichomoniasis, a genitourinary parasitic infection responsible for an estimated 156 million new cases annually (Harfouche et al., 2024; World Health Organization (WHO), 2024). While this disease is generally asymptomatic in men, it may manifest in women through symptoms such as vaginal discharge and vulvar pruritus (Harfouche et al., 2024; WHO & Pan American Health Organization, 2024.). Trichomoniasis is primarily sexually transmitted, with the protozoan existing predominantly in the trophozoite form, where the flagella are externalized (Benchimol et al., 2022; Harfouche et al., 2024). While trichomonads do not present a true cyst form, endoflagellar forms have been observed (Benchimol et al., 2022).

Pharmacological treatment for both parasitic infections relies primarily on metronidazole (a 5-nitroimidazole) (Benchimol et al., 2022; The Medical Letter, 2013). In the case of giardiasis, albendazole (a benzimidazole) is also frequently employed as a therapeutic agent (Benchimol et al., 2022; Solaymani-Mohammadi et al., 2010). However, the current treatment options exhibit certain limitations in terms of both efficacy and tolerability. For instance, metronidazole, which serves as the first-line treatment for both infections, is associated with adverse effects including nausea, vomiting, and metallic taste sensation (Benchimol et al., 2022; Leung et al., 2019; Solaymani-Mohammadi et al., 2010). Furthermore, treatment failure and reinfection are not uncommon (Upcroft and Upcroft 2001). Consequently, given the significant adverse effects of these drugs and the emergence of drug-resistant strains, the development of new antiparasitic drugs is necessary to provide better alternative treatment options for patients (Zheng et al. 2014; Hernández-Ochoa et al. 2022).

In vitro models of parasites play crucial roles in the search for therapeutic alternatives for the treatment of parasitic diseases, as they facilitate the identification of new mechanisms of action and therapeutic targets. These models are invaluable for studying parasite–host interactions and investigating parasite virulence and pathogenesis mechanisms (Sutrave & Richter 2021). In addition, *in vitro* culturing of trophozoites remains the standard laboratory tool for drug discovery, resistance monitoring and fundamental research (Zheng et al. 2014). In this context, assessment of cell viability in parasites is crucial for evaluating the efficacy of antiparasitic drugs and developing new treatment strategies.

Despite the pressing need for improved treatment options, the choice of an appropriate method for assessing the *in vitro* viability of *G. lamblia* and *T. vaginalis* in the presence of therapeutic compounds remains a challenge. Over the years, multiple methods have been developed and employed to assess the viability of these parasites. These methods range from traditional techniques such as culturing, microscopy, and dye exclusion to more modern molecular and imaging-based approaches (Kulakova et al. 2014; Singh et al. 2016; Hernández-Ochoa et al. 2022). However, the diversity of available methods often leads to inconsistencies in results and difficulties in comparing studies, making it challenging to establish a standardized approach for assessing viability. A better understanding of these methods could improve the discovery of new treatment strategies and provide a valuable resource for researchers, clinicians, and public health professionals.

Therefore, the primary aim of this study was to provide a comprehensive overview of commonly used methods for assessing the viability of *G. lamblia* and *T. vaginalis* trophozoites in the presence of compounds with antiparasitic potential. Additionally, this study aims to propose a standardized viability assay for susceptibility testing in *G. lamblia* and *T. vaginalis*.

3.3. Materials and methods

The current systematic review was conducted according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA 2020) statement (Page et al. 2021). The SPIDER tool adapted from Cooke et al. (2012) was employed to formulate the following research question: "What methodologies are most commonly employed in *in vitro* studies for assessing the antigiardial and antitrichomonal activity of compounds with potential therapeutic activity?" (Table 8).

Table 8 – Description of the employed SPIDER tool adapted from Cooke et al. (2012).

SPIDER Tool	
S – Sample	*In vitro* studies assessing compounds against *G. lamblia* and *T. vaginalis*
PI – Phenomenon of Interest	Methodologies employed for assessing antigiardial and antitrichomonal activity
D – Design	Experimental studies
E – Evaluation	Measures of activity (viability, motility, and growth inhibition)
R – Research type	Methodological studies, experimental research

3.3.1. Information sources and search strategy

A literature search was conducted across the MEDLINE, ScienceDirect, and Web of Science databases using the following search terms: "*in vitro*", "method", "susceptibility", "viability", "sensitivity", "giardia" and "trichomonas". The search terms were combined using Boolean operators "AND" and "OR". The following search equation was used: "*in vitro*" AND "method" AND ("susceptibility" OR "viability" OR "sensitivity") AND ("giardia" OR "trichomonas"). In the ScienceDirect database, the search was based on title, abstract or author-specified keywords. In MEDLINE, the available MeSH terms were used. The databases were accessed on 21 August 2024.

3.3.2. Eligibility criteria

Studies eligible for inclusion were research articles, written in English, Portuguese, or Spanish. These studies were specifically required to assess *in vitro* methods to evaluate the viability of *G. lamblia* and *T. vaginalis* trophozoites. Studies addressing cyst viability or lacking explicit specification of the method employed were excluded, as were reviews and meta-analyses. No restrictions were imposed regarding the timeframe for the search.

3.3.3. Quality assessment

A critical appraisal was used to assess the quality of the studies included in the review, through the development of a checklist adapted from McConn et al. (2024). The checklist included parameters such as a clearly stated study objective aligned with assessing antigiardial and antitrichomonal activity, a thorough description of the *in vitro* assay methodologies, adequate and clearly described sample sizes and use of controls, well-defined and appropriate outcome measures for evaluating activity, appropriate data analysis methods with clear presentation and statistical analysis of results, sufficient methodological detail for reproducibility and replicability, identification and mitigation of potential sources of bias, and conclusions supported by the obtained data. Eight parameters, in the form of questions, were scored on a yes-no basis and "not applicable or not sure" (Table 9). The articles were grouped by the number of "yes" by quality into a scale: 1-2 unsatisfactory; 3-4 satisfactory; 5-6 good; and 7-8 excellent.

Table 9 – Quality assessment criteria used for the evaluation of the articles adapted from McConn et al. (2024).

Questions	Criteria		
1. Is the study objective clearly stated, and does it align with the research question of assessing antigiardial and/or antitrichomonal activity?	Yes	No	Not applicable/not sure
2. Are the sample size and use of controls adequate and clearly described?			
3. Is the methodology for the *in vitro* assays thoroughly described, including detailed protocols and conditions?			
4. Are the outcome measures (e.g., viability, motility, growth inhibition) clearly defined and appropriate for assessing antigiardial and antitrichomonal activity?			
5. Is the data analysis method appropriate, and are the results presented clearly with adequate statistical analysis?			
6. Are the methods and results reproducible and replicable, with sufficient detail provided to allow replication by other researchers?			
7. Does the study address potential sources of bias and confounding factors that could affect the results?			
8. Are the conclusions supported by the data, and do they discuss the implications for future research or clinical application?			

3.4. Results

3.4.1. Characteristics of the retrieved records

The electronic search yielded a total of 148 articles, with 56 entries retrieved from MEDLINE, 23 from ScienceDirect, and 69 from Web of Science (Figure 3). These sets of records were downloaded from each respective database and then integrated into the Rayyan platform (Ouzzani et al. 2016). This consolidation served the dual purpose of removing duplicate records and facilitating the retrieval of pertinent articles. Following the elimination of duplicates, a total of 110 studies remained for further evaluation. The titles and abstracts of all identified studies were independently examined by two reviewers, according to predefined inclusion and exclusion criteria. Disagreements between the researchers were resolved through consensus discussions, obviating the need for a third opinion. Records that were evidently irrelevant were excluded. In cases where the abstract and/or title did not provide sufficient information to comply with the inclusion criteria, the full text of the report was obtained for thorough evaluation. Consequently, 36 studies were selected for full-text reading, and these were independently assessed by the same two reviewers. Articles that did not meet all the inclusion criteria after the full-text assessment (n=4) were excluded from further examination. Figure 3 illustrates and summarizes the complete study selection process.

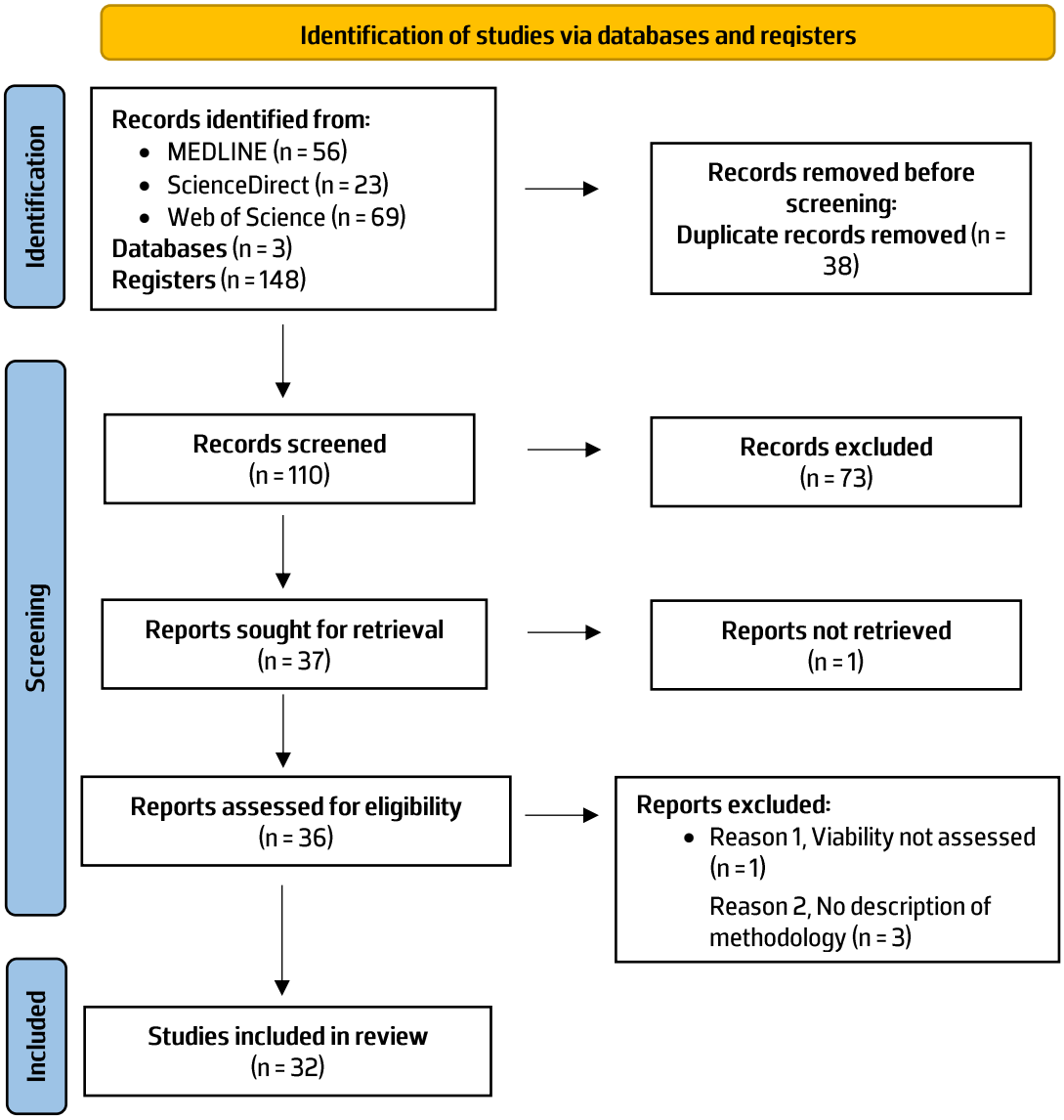

Figure 3 – Flowchart of the selection procedure adapted from the PRISMA 2020 statement (Page et al. 2021).

3.4.2. Quality assessment

To minimize the risk of bias, an assessment of the quality of the articles included in the review was conducted through the development of an 8-question checklist. The studies included in the review (n=32) were evaluated based on this checklist (Figure 4). Of these, 46.9% were classified as good, followed by excellent (37.5%) and satisfactory (15.6%). No articles were classified as unsatisfactory; therefore, no articles were excluded.

Article	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Overall
Afzan et al. 2012	✓	✓	✓	✓	✓	✓	✗	✗	
Aldrete et al. 2005	✓	✓	✓	✓	✓	✓	✗	✓	
Argüello-García et al. 2004	✓	✓	✓	✓	✓	✓	✓	✓	
Barbosa et al. 2014	✓	✓	✓	✓	✓	✓	✗	✓	
Bénéré et al. 2007	✓	✓	✓	✓	✓	✓	✓	✓	
Boreham et al. 1984	✓	✓	✓	✓	✗	✓	✓	✓	
Bromke et al. 1995	✓	✓	✓	✓	✗	✓	✗	✓	
Chaudhari and Singh 2011	✓	✓	✓	✗	✗	✓	✗	✓	
Chen et al. 2011	✓	✓	✓	✓	✓	✓	✗	?	
Chen et al. 2023	✓	✓	✓	✓	✓	✓	✗	✓	
Crouch et al. 1986	✓	✓	✓	✓	✓	✓	✗	✗	
Cruz et al. 2003a	✓	✓	✓	✓	✓	✓	✗	✓	
Cruz et al. 2003b	✓	✓	✓	✓	✓	✓	✗	✗	
Downey et al. 2009	✓	✓	✓	✓	✓	✓	✗	✓	
Escribano et al. 2012	✓	✓	✓	✓	✓	✓	✓	✓	
Gadelha et al. 2020	✓	✓	✓	✓	✓	✓	✗	✗	
Gordts et al. 1985	✓	✓	✓	✓	✗	✓	✗	✓	
Hezarjaribi et al. 2022	✓	?	✓	✓	✗	✓	✗	✓	
Hounkong et al. 2011	✓	✓	✓	✓	✓	✓	✗	✓	
Kang et al. 1998	✓	✓	✓	✓	?	✓	✓	✓	
Karami et al. 2019	✓	✓	✓	✓	✓	✓	✓	✓	
Karami et al. 2023	✓	✓	✓	✓	✓	?	✗	✓	
Lara-Díaz et al. 2008	✓	?	✓	?	✓	?	✗	✓	
Meri et al. 2000	✓	✓	✓	✓	?	✓	✗	✓	
Naidoo et al. 2013	✓	?	✓	✓	✗	✓	✓	✓	
Osmari et al. 2020	✓	?	✓	✓	✓	✓	✗	?	
Özel et al. 2024	✓	✓	✓	✓	?	✓	✗	✓	
Ponce-Macotela et al. 2001	✓	✓	?	✓	✓	✓	✗	✓	
Smith and Domenico 1980	✓	✓	✓	✓	✗	?	✗	✓	
Tiwari et al. 2008	✓	✓	?	✓	?	✓	✗	✓	
Wright et al. 1992	✓	✓	✓	✓	?	✗	✗	✗	
Yadegari et al. 2022	✓	✓	✓	?	✓	✗	✗	✓	

Caption

✓ Yes

? Not applicable/ not sure

✗ No

Excellent

Good

Satisfactory

Unsatisfactory

Figure 4 – Quality assessment of the reviewed articles considering the questions (Q1 to Q8) formulated in Table 9.

3.4.3. Parasite viability and identification of methods

The search identified a variety of viability assays for both parasites, which were grouped into categories based on principles of viability measurement, including colorimetric, fluorometric, luminescence, microbiological, morphophysiological, and radioisotopic methods. The morphophysiological category was established to include assays that evaluate changes in cell morphology and physiological properties, such as adherence and motility. The advantages and disadvantages of each method, as well as the respective parameters used to evaluate cell viability, were assessed.

3.4.4. Viability assays for *G. lamblia*

A total of 21 viability assays were identified for *G. lamblia* (Table 10). The category with the highest number of assays is the colorimetric group, which includes 8 assays, followed by the morphophysiological group, which comprises 5 assays. The most frequently cited methods were the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the adherence inhibition assay, the [^{3}H]-thymidine incorporation assay and the cell count assay.

The MTT assay involves incubating cells with MTT for a few hours, during which mitochondrial dehydrogenases in viable cells reduce tetrazolium into purple formazan crystals. The water-insoluble formazan crystals are then solubilized using a solvent such as Dimethyl sulfoxide (DMSO). The absorbance of the resulting MTT-formazan solution is measured using a microplate reader at a wavelength around 570 nm. The measured optical density values obtained are indicative of the concentration of formazan, which correlates with the number of viable cells and their metabolic activity (Ghasemi et al., 2021).

The adherence inhibition assay involves incubating *G. lamblia* trophozoites with a test substance and subsequently evaluating their adherence to a substrate, such as a cell monolayer or a plastic surface (Machado et al. 2010). Adherence serves as a crucial viability indicator, with adherence levels quantified by enumerating the adhered trophozoites or measuring the optical density of stained trophozoites (Morgan et al. 1993).

The [^{3}H]-thymidine assay involves labelling cells with radioactive [^{3}H]-thymidine, which is then incorporated into their deoxyribonucleic acid (DNA). The amount of [^{3}H]-thymidine integrated into the DNA can be quantified via scintillation counting or autoradiography. This assay is based on the principle that actively dividing cells incorporate more [^{3}H]-thymidine into their DNA than non-dividing or declining cells (Kang et al. 1998).

Table 10 – Assays for viability assessment of *G. lamblia* trophozoites.

Type of assay	Assay name	Parameter	Cited by	Advantages	Disadvantages	References
Colorimetric	Crystal violet	IC_{50}	Hounkong et al. (2011)	• Shows a more satisfactory OD reading than eosin.	• Has tendency to stain the tissue culture plate.	Wright et al. (1992); Kang et al. (1998); Bénéré et al. (2007); Hounkong et al. (2011)
	Methylene blue	IC_{50}		• Shows a more satisfactory OD reading than eosin.	• Removal of culture medium is necessary; • Requires multiple wash steps.	
	Eosin	IC_{50}	Hounkong et al. (2011); Yadegari et al. (2022)	–	• Less satisfactory OD reading than crystal violet and methylene blue.	
	MTS/PMS	IC_{50}	Gadelha et al. (2020)	–	• Removal of culture medium is necessary; • Requires multiple wash steps.	
	MTT	IC_{50}	Argüello-García et al. (2004); Bénéré et al. (2007); Ponce-Macotela et al. (2001)	–	• Time-consuming; • Less suitable for viability determination of low trophozoite burdens; • Needs an additional extraction step (dissolution of crystals with DMSO); • Replacement of the medium with PBS may be necessary to prevent spontaneous conversion of MTT; • The washing step disturbs the attachment of the parasite to the plate.	
	NPR	IC_{50}	Kang et al. (1998)	•Simple; • Determined directly by the change in absorbance; • Both continuous and stopped assays are feasible.	–	
	Trypan blue	ID_{50}	Boreham et al. (1984); Bénéré et al. (2007)	• Simple; • Widely used.	• Laborious; • Time-consuming; • Requires a microscopic examination to count cells with a haemocytometer.	
	XTT	IC_{50}	Wright et al. (1992); Bénéré et al. (2007)	• Readily reduced by the parasite; • Measured directly by absorption.	• Time-consuming; • Replacement of the medium with PBS may be necessary to prevent spontaneous conversion of XTT; • The washing step disturbs the attachment of the parasite to the plate.	

Type of assay	Assay name	Parameter	Cited by	Advantages	Disadvantages	References
Fluorometric	Resazurin or Alamar blue	IC_{50}	Bénéré et al. (2007)	· Simple; · Rapid; · Sensitive; · Reliable; · Cost-effective; · Reproductible; · Easy endpoint reading; · Dynamic follow-up experiments remain possible.	· Replacement of the medium with PBS may be necessary to prevent spontaneous conversion of resazurin; · The washing step disturbs the attachment of the parasite to the plate; · Dynamic follow-up experiments remain possible.	Argüello-García et al. (2004); Bénéré et al. (2007); Downey et al. (2009); Chen et al. (2011); Hounkong et al. (2011); Barbosa et al. (2014)
	FDA-PI	IC_{50}	Argüello-García et al. (2004)	· Fast; · Cost-effective; · Efficient; · Can be evaluated by flow cytometry.	· Requires multiple wash steps; · Requires a microscopic examination to count cells.	
	PI	IC_{50}	Barbosa et al. (2014)	· Accurate; · Simple; · Automated analysis; · Reproductible; · Time-saving; · More optimal than SYBR Green I staining; · Growth inhibition assessed through the adherence properties of the parasite; · Can be evaluated by flow cytometry.	-	
	SYBR Green I	IC_{50}	Downey et al. (2009)	· Interacts with nucleic acids, binding directly to DNA or RNA.	· Requires multiple wash steps; · Replacement of culture medium is necessary; · Less optimal than the PI screen; · Can not be used to distinguish between live and dead trophozoites.	

Type of assay	Assay name	Parameter	Cited by	Advantages	Disadvantages	References
Morpho-physiological	Adherence Inhibition	IC_{50}	Crouch et al. (1986); Cruz et al. (2003a,b); Barbosa et al. (2014)	· Simple; · Provides insights into mechanisms of drug action; · Preferred for drugs with a mechanism of action similar to benzimidazoles; · Easier to perform and less time consuming than the multiplication method.	· Laborious; · Time-consuming; · Subjective; · Only accurate when cells are truly confluent; · Difficult to assay for several compounds at the same time; · Requires a microscopic examination to count adherent cells.	Crouch et al. (1986); Kang et al. (1998); Cruz et al. (2003b); Argüello-García et al. (2004); Hounkong et al. (2011)
	Growth Inhibition or Multiplication Method	IC_{50}	Crouch et al. (1986); Cruz et al. (2003b)	· Reliable; · Sensitive for high inhibitory concentrations of benzimidazoles.	· Inactivation of parasites is necessary; · Hard to perform; · Time-consuming; · Requires a microscopic examination to count cells with a haemocytometer.	
	Cell Count	IC_{50} %GI	Bénéré et al. (2007); Lara-Díaz et al. (2008); Hounkong et al. (2011)	-	· Laborious; · Time-consuming; · Subjective; · Difficult to assay for several compounds at the same time.	
	Cell Morphology	IC_{50}	Argüello-García et al. (2004)	· Sensitive; · Gives direct measurements of cell viability; · Useful to evaluate the effect of benzimidazoles and 5-nitroimidazoles.	· Laborious; · Time-consuming; · Requires a microscopic examination to count cells with a haemocytometer; · Subjective; · Difficult to assay for several compounds at the same time.	
	Cell Motility	MIC	Boreham et al. (1984)	-	· Unreliable; · Insensitive; · Lacks standardization; · Does not consider that organisms may be reproductively non-viable while still showing flagellar activity.	

Type of assay	Assay name	Parameter	Cited by	Advantages	Disadvantages	References
Luminescence	ATP Content	IC_{50}	Chen et al. (2011)	· Nonbiased; · Homogeneous format (amenable to high-throughput screens); · Reproductible; · Dynamic follow-up experiments remain possible.	-	Chen et al. (2011)
Micro-biological	MMSM or CGSSM	MIC	Gordts et al. (1985)	-	· Impracticable; · Requires large volumes of culture medium; · Expense of materials; · Problems of replication; · Plating efficiency of only 25%; · Requires a microscopic examination to count cells with a haemocytometer.	Gordts et al. (1985); Crouch et al. (1986); Argüello-García et al. (2004)
	SCLM	IC_{50}	Argüello-García et al. (2004)	· Highly sensitive method for assessing the effects of 5-nitroimidazoles and benzimidazoles.	· Indirect; · Time-consuming; · Requires a microscopic examination to count cells with a haemocytometer. · Efficiently replaced by other viability assays.	
Radioisotopic	[^{3}H]-thymidine	ID_{50} IC_{50}	Boreham et al. (1984); Argüello-García et al. (2004); Downey et al. (2009)	· Simple; · Reliable; · Nonbiased; · Measures the target for three of the drugs commonly used in the treatment of giardiasis.	· Laborious; · Time-consuming; · Incorporation of [^{3}H]-thymidine into intact cells induces cell separation; · Requires multiple wash steps; · Difficult to assay for several compounds at the same time.	Boreham et al. (1984); Kang et al. (1998); Chen et al. (2011); Hounkong et al. (2011)

Caption: ATP – Adenosine triphosphate; CGSM – Cell Growth in Semi-solid Medium; DMSO – Dimethyl sulfoxide; FDA/PI – Fluorescein Diacetate/Propidium Iodide; IC_{50} - Half Maximal Inhibitory Concentration; ID_{50} - Median Infective Dose; %GI – Growth Inhibition; MIC - Minimum Inhibitory Concentration; MMSM – Macrodilution Method in Semi-solid Medium; MTS/PMS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/Phenazine Methosulfate; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NPR - 4-nitrophenyl b-dribofuranoside; OD – Optical Density; PBS - Phosphate-buffered saline; PI - Propidium Iodide; SCLM - Subculture in Liquid Medium; SYBR Green I: 2-[4-(2-hydroxyethyl)-1-piperazinyl]-N,N-dimethyl-6-(4-nitrophenylamino)-3-hydroxy-2,7-naphthalenedisulfonamide; XTT - 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

3.4.5. Viability assays for *T. vaginalis*

A total of 8 viability assays were identified for *T. vaginalis* (Table 11). The category with the highest number of assays is the colorimetric group, which includes 3 assays. The most frequently cited methods include the trypan blue assay, motility assessment, resazurin assay, and cell count. The trypan blue assay involves mixing a cell suspension with the dye, with viable cells identified by counting the unstained cells under a microscope or automated counter. This method relies on the principle of dye exclusion, where viable cells remain unstained due to their intact membranes, whereas dead cells retain the blue dye due to compromised membrane integrity (Ramirez et al. 2010; Parboosing et al. 2016; Aslantürk 2017). The motility assay relies on visual examination of cell motility using an inverted microscope (Meri et al. 2000). Typically, all motile cells, including stationary ones displaying movement of flagella and/or undulating membranes, are counted (Bromke et al. 1995; Chaudhari and Singh 2011). Some authors solely observe motility, while others quantify motile cells using a haemocytometer (Bromke et al. 1995; Meri et al. 2000; Chaudhari and Singh 2011). The resazurin assay involves the conversion of the non-fluorescent blue dye, resazurin, to the pink fluorescent resorufin by the mitochondrial enzymes of viable cells (Rampersad 2012; Parboosing et al. 2016; Aslantürk 2017). This change from an oxidized state to a reduced state allows for quantitative colorimetric and/or fluorometric readings, with the latter being more sensitive. It also permits qualitative detection through a visible colour change indicating the presence or absence of viable cells (Rampersad 2012; Aslantürk 2017).

Table 11 – Assays for viability assessment of *T. vaginalis* trophozoites.

Type of assay	Assay name	Parameter	Cited by	Advantages	Disadvantages	References
Colorimetric	INT	–	Naidoo et al. (2013)	• Allows qualitative viability assessment.	• Time-consuming (needs six hours to act).	Aldrete et al. (2005); Tiwari et al. (2008); Afzan et al. (2012); Naidoo et al. (2013); Osmari et al. (2020); Hezarjaribi et al. (2022)
	Modified Field Stain	–	Afzan et al. (2012)	• Provides visualization of internal morphological changes.	• Requires a microscopic examination to count cells with a haemocytometer.	
	Trypan blue	MIC IC_{50}	Tiwari et al. (2008); Afzan et al. (2012); Osmari et al. (2020); Hezarjaribi et al. (2022)	• Simple; • Widely used.	• Laborious; • Time-consuming; • Requires a microscopic examination to count cells with a haemocytometer.	
Fluorometric	Resazurin or Alamar blue	EC_{50} MIC IC_{50} %GI	Aldrete et al. (2005); Escribano et al. (2012)	• Allows qualitative and quantitative viability assessment; • Cost-effective; • Economic; • May be used in combination with fluorometry; • Non-subjective; • Practical; • Reliable; • Sensitive; • Time-saving.	• Replacement of the medium with PBS may be necessary to prevent spontaneous conversion of resazurin.	Aldrete et al. (2005); Escribano et al. (2012); Chen et al. (2023)
	SYBR Green I	IC_{50}	Chen et al. (2023)	• Good substitute for ethidium bromide; • Interacts with nucleic acids, binding directly to DNA or RNA; • Cost-effective; • Time-saving; • Labor-saving; • Efficient; • Non-subjective.	• Modification of cell culture may be required.	

Type of assay	Assay name	Parameter	Cited by	Advantages	Disadvantages	References
Microbiological	Disk Broth Method	MIC	Smith & Domenico (1980)	• Simple; • Reliable for detection of metronidazole-resistant strains.	• Requires a microscopic examination to count cells with a haemocytometer.	Smith & Domenico (1980)
Morpho-physiological	Cell Count	MLC IC_{50} %GI	Karami et al., (2019, 2023); Lara-Díaz et al. (2008)	-	• Laborious; • Time-consuming; • Insensitive; • Subjective (dependent on the experience of the observer).	Bromke et al. (1995); Meri et al. (2000); Aldrete et al. (2005); Chaudhari & Singh (2011); Escribano et al. (2012)
	Cell Motility	MLC MIC	Bromke et al., (1995); Chaudhari & Singh (2011); Meri et al., (2000); Özel et al. (2024)	-	• Requires a microscopic examination to count cells with a haemocytometer; • Laborious; • Time-consuming.	

Caption: DNA - Deoxyribonucleic acid; EC_{50} - Half Maximal Effective Concentration; IC_{50} - Half Maximal Inhibitory Concentration; INT - p-iodonitrotetrazolium violet; %GI – Growth Inhibition; MIC - Minimum Inhibitory Concentration; MLC - Minimum Lethal Concentration; PBS – Phosphate-buffered saline; RNA - Ribonucleic acid; SYBR Green I - 2-[4-(2-hydroxyethyl)-1-piperazinyl]-N,N-dimethyl-6-(4-nitrophenylamino)-3-hydroxy-2,7-naphthalenedisulfonamide.

3.4.6. Viability assays common to both parasites

Only five viability assays were common to both *G. lamblia* and *T. vaginalis*: a colorimetric method (dye exclusion test with trypan blue), two fluorometric methods (resazurin and SYBR green I), and two morphophysiological methods (cell count and cell motility). Among these methods, the cell counting assay was the most frequently cited. This method involves loading a cell suspension onto either a counting chamber or a haemocytometer, followed by microscopic examination. To distinguish between viable and non-viable cells, the incorporation of specific dyes is customary (Burleson et al. 1992). Alternatively, automated cell counting systems can be employed, offering benefits such as reduced analyst-dependent variability and shorter analytical times when compared to traditional manual methods (Peli et al. 2023).

3.5. Discussion

The present systematic review aimed to evaluate the methodologies employed for assessing the antigiardial and antitrichomonal activity of compounds *in vitro*, with a particular focus on identifying and recommending a standardized protocol. Our analysis highlights significant variability in the assays used, with notable differences in reagents, experimental conditions, and outcome measures across studies.

Toxicity is commonly expressed in terms of the Half Maximal Inhibitory Concentration (IC_{50}), which represents the concentration of a test substance that reduces cell viability to 50% when compared to untreated control cells (Parboosing et al. 2016). As expected, the IC_{50} was the most frequently cited parameter for evaluating cell viability in the collected articles. It is crucial to emphasize that IC_{50} values obtained through different assays may not be directly comparable and are typically dependent on the specific cell type and strain (Parboosing et al. 2016). Additionally, it is important to highlight that using different parameters within the same viability assays can yield in different sensitivities and responses, potentially influencing the interpretation and comparison of results across different studies (Aslantürk 2017). This underscores the necessity to standardize the parameter for evaluating toxicity.

One significant observation arising from the analysis of the collected articles is the acknowledgement of potential subjectivity introduced by qualitative measurements of cell viability, as occasionally employed in the MTT and resazurin assays (Hounkong et al. 2011). Subjectivity in viability assessment can hinder the reproducibility and reliability of results. Additionally, it was noted that, in certain instances, the removal of the test compound becomes necessary to prevent reagent depletion or interfere with its metabolic reduction, potentially leading to inaccurate measurements (Wright et al. 1992). Moreover, numerous colorimetric, morphophysiological and microbiological methods require a subsequent microscopic examination for cell counting using a haemocytometer, introducing additional subjectivity and errors while also prolonging the process. The use of glucose to enhance the sensitivity of the resazurin assay has yielded varied findings within the scientific literature. While some authors have reported that the addition of glucose can increase sensitivity, others have observed no significant impact on the assay's performance (Bénéré et al. 2007; Escribano et al. 2012). Furthermore, a disparity exists among researchers regarding the necessity of removing the culture medium before undertaking the resazurin assay. Some researchers advocate for the complete removal of the medium to avert potential interference, while others prefer to modify

the medium through dilution or alternative means (Bénéré et al. 2007; Escribano et al. 2012). On the other hand, some researchers opt not to alter or remove the culture medium (Aldrete et al. 2005). These divergent methodological approaches underscore the imperative need for a standardized protocol to ensure result consistency, thereby facilitating meaningful comparisons across numerous studies.

In the context of selecting cytotoxicity and cell viability assays, considerations such as availability in the laboratory, test compounds, detection mechanism, specificity, and sensitivity should be considered (Aslantürk 2017). Furthermore, while some studies express a preference for certain viability assessment methods over others, our analysis reveals that the choice of method was often influenced by factors such as the availability of resources, the research setting, and the specific research question. For instance, in resource-limited settings, where sophisticated equipment and reagents may not be readily available, direct microscopic examination remains a practical and valuable tool for assessing viability. However, in clinical diagnostic laboratories with access to advanced technology, molecular assays, such as fluorometric and colorimetric methods, might be the preferred choice. Therefore, researchers should consider the context in which they are working and the specific characteristics of *G. lamblia* and *T. vaginalis* when selecting a viability assay.

The resazurin assay has proven to be a reliable, sensitive, and cost-effective method for assessing the viability of *G. lamblia* and *T. vaginalis* trophozoites. To standardize this method across different laboratories, we suggest performing the assay in a 96-well plate format, where the volume of resazurin added should correspond to 10% of the final well volume at a concentration of 0.1 – 1 mg/ml (Duarte et al., 2009). The inoculum should contain 10^4 to 10^6 trophozoites per well and the incubation time should be 1 hour for *T. vaginalis* and 2-4 hours for *G. lamblia* at 37ºC under low oxygen conditions (Bénéré et al., 2007; Escribano et al., 2012; Wongstitwilairoong et al., 2023). In each plate, background (medium only), vehicle (DMSO (≤1%) or other), and no vehicle (parasites and media) should be included as control wells (Wongstitwilairoong et al. 2023). Measurements of resazurin reduction can be conducted either by colorimetry (qualitative) or fluorimetry (quantitative); however, greater sensitivity is achieved using the fluorescent method. The fluorescence should be measured at 535-560 nm excitation and 590 nm emission (Duarte et al. 2009; Escribano et al. 2012). This method is non-radioactive, rapid, one-step process, that requires no sophisticated equipment. Additionally, the microplate format offers possibilities for automation, making it an excellent asset for

screening large compound libraries in new drug discovery efforts (Bénéré et al. 2007; Escribano et al. 2012). Standardizing the resazurin assay in this manner will increase the reproducibility and comparability of the results, ultimately advancing research on *G. lamblia* and *T. vaginalis*. In the absence of a fluorimeter, the MTT assay presents a valid alternative for assessing the viability of both parasites (Singh et al., 2016). This method requires an absorbance reader, such as a spectrophotometer or a simple microplate reader, which makes it a more cost-effective option. Although the MTT assay has some disadvantages, it remains a reliable and widely used method for evaluating cell viability due to its simplicity and affordability (Ghasemi et al., 2021).

3.6. Conclusion

The present systematic review highlights the diversity of viability assessment methods available for *G. lamblia* and *T. vaginalis*. This analysis underscores the importance of understanding the strengths and weaknesses of each method, which is crucial for informed decision-making when conducting research or diagnostic testing with these parasites.

For *G. lamblia*, the MTT assay, adherence inhibition assay, and [^{3}H]-thymidine incorporation assay were the most frequently cited. These methods offer distinct advantages, such as the simplicity and cost-effectiveness of the MTT assay, the functional insights provided by the adherence inhibition assay, and the measurement of cellular proliferation with the [^{3}H]-thymidine incorporation assay. However, limitations include potential interference from cellular debris, the need for specific equipment, and additional cell washing steps.

For *T. vaginalis*, the trypan blue exclusion assay, motility assessment, and resazurin assay were commonly used. While the trypan blue assay is simple, it may not always effectively differentiate between live and dead cells, particularly when cell membrane integrity is compromised. The motility assessment offers functional insights but is labour-intensive and subjective. The resazurin assay is highly sensitive and can detect small changes in viability, though potential interference from the media or test compounds may lead to inaccurate results.

The analysis also highlighted potential challenges introduced by qualitative measurements instead of quantitative ones in assays such as MTT and resazurin, emphasizing the need for meticulous consideration of subjectivity in viability assessment. The necessity of test compound removal to avoid interference and inaccurate measurements, as well as the controversial findings regarding the use of glucose in enhancing resazurin assay sensitivity, underscore the importance of standardized protocols for result consistency.

Among the methods reviewed, the fluorometric resazurin assay emerged as the most suitable for assessing trophozoite viability in both parasites due to its cost-effectiveness, reliability, and ease of use. Further research and standardization of viability assessment methods are essential to the understanding of drug susceptibility and the developing of effective treatment strategies for these parasitic infections.

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4. Chapter 3 – Evaluation of the antiparasitic potential of selected medicinal plants based on Portuguese ethnobotany in *Giardia lamblia* and *Trichomonas vaginalis*

4.1. Abstract

Parasitic infections remain a significant challenge to global public health, particularly in resource-poor settings. Given the significant adverse effects associated with antiparasitic drugs and the emergence of drug-resistant strains, the development of more effective, selective, and less toxic drugs is necessary to provide better alternative treatment options for patients. Ethnopharmacology, which explores the traditional use of medicinal plants, serves as a valuable resource for discovering new compounds with therapeutic potential. This study aimed to assess the antiparasitic potential of 11 medicinal plants, comprising 22 extracts, which are traditionally used by the Portuguese community to treat parasitic diseases, against *Giardia lamblia* and *Trichomonas vaginalis* trophozoites. The research involved characterizing cell growth and viability assays to determine the optimal conditions for susceptibility assays. The *in vitro* susceptibility of both aqueous and methanolic extracts was performed using serial dilutions ranging from 5 µg/ml to 2000 µg/ml. The inhibitory effect of metronidazole was assessed, revealing IC_{50} values ranging from 12.58 to 13.38 µM for *G. lamblia* and 24.37 to 25.45 µM for *T. vaginalis*. Additionally, the medicinal plant extracts demonstrated low activity against both *G. lamblia* and *T. vaginalis* trophozoites, with IC_{50} values greater than 500 µg/ml. However, three aqueous extracts (*Artemisia absinthium* L., *Chenopodium ambrosioides* L. and *Cucurbita pepo* L.) exhibited promising potential in cell proliferation of *G. lamblia* at higher concentrations. The variability in trophozoite growth inhibition observed across previous studies and the present investigation underscores the influence of multiple variables affecting the bioactivity and efficacy of the extracted compounds, highlighting the need for standardized methodologies in antiprotozoal research.

Keywords: Ethnobotanic studies; *Giardia lamblia;* Medicinal plants; *Trichomonas vaginalis.*

4.2. Introduction

Giardiasis is an intestinal parasitic infection caused by the flagellated protozoan parasite *Giardia lamblia*. The parasite is transmitted primarily through the faecal-oral route, often through the ingestion of contaminated water and food. Transmission can also occur through person-to-person contact, while animal-to-person transmission is relatively rare. Giardiasis is particularly common in areas with poor sanitation and limited water treatment facilities (Leung et al., 2019). In 2019, 18.004 confirmed giardiasis cases were reported in the European Union and European Economic Area (European Centre for Disease Prevention and Control (ECDC), 2022). Despite being a notifiable disease, these numbers may not accurately reflect the true prevalence due to the fact that a significant proportion of infections, ranging from 50 to 75%, are asymptomatic. Those who do exhibit symptoms, often present with acute or chronic diarrhoea, along with other chronic gastrointestinal symptoms (ECDC, 2022; Leung et al., 2019).

Trichomoniasis is a sexually transmitted genitourinary parasitic infection caused by *Trichomonas vaginalis*, another flagellated protozoan parasite (Edwards et al., 2014). Infections of the female genital tract can cause a range of symptoms, including vaginitis and cervicitis. Infections in males are generally asymptomatic, although mild urethritis or prostatitis can occur (Edwards et al., 2014; World Health Organization (WHO) & Pan American Health Organization (PAHO), 2024). Trichomoniasis is the most prevalent nonviral sexual infection, with an estimated 156 million new cases annually (WHO, 2023). Currently, trichomoniasis is not categorized as a reportable infection, so prevalence and infection rates have to be estimated due to the absence of available case reporting data (Edwards et al., 2014). Metronidazole is commonly used to treat both infections (The Medical Letter, 2013). This chemotherapeutic antiparasitic agent is usually associated with adverse effects including nausea, vomiting, and metallic taste sensation (Leung et al., 2019; Solaymani-Mohammadi et al., 2010). Treatment failure and reinfection are also common (Upcroft & Upcroft, 2001). Given the significant adverse effects of the antiparasitic drugs and the emergence of drug-resistant strains, the development of more effective, selective, and less toxic drugs is necessary to provide better alternative treatment options for patients (Hernández-Ochoa et al., 2022; Zheng et al., 2014).

Despite the advances in the development of new drugs of synthetic origin, natural molecules obtained from medicinal plants and their derivatives remain valuable sources of new therapeutic compounds (Núñez et al., 2023; Ramirez-Moreno et al., 2017). For centuries, plants with therapeutic properties have been identified and used in traditional medicine for the treatment of common human diseases and the control of protozoan parasites and their vectors. Compared with synthetic alternatives, medicinal plant-based antimicrobials represent a significant reservoir of pharmaceuticals, offering potential solutions with fewer side effects (Das et al., 2010). A recent study identified medicinal plants within the Portuguese community's traditional practices for treating parasitic infections (data not published). In the context of these findings, this research aims to assess the antiparasitic potential of 11 medicinal plants (22 extracts), which are traditionally used by the Portuguese community to treat parasitic diseases, against *G. lamblia* and *T. vaginalis* trophozoites. Additionally, this research aims to identify the most practical and feasible viability assay for implementation in routine laboratory tests, facilitating the assessment of parasite susceptibility to new therapeutic compounds and enabling large-scale screening.

4.3. Materials and methods

The chemotherapeutic agent used was metronidazole (Sigma). A stock solution of 10 mg/ml metronidazole was prepared with ultrapure water. For the preparation of the two culture media, the following reagents were used: bacteriological agar (VWR Chemicals), bovine bile (Sigma), ferric ammonium citrate (MP Biomedicals), glucose anhydrous (Labkem), L-ascorbic acid (Thermo Fisher Scientific), L-cysteine hychloride monohydrate (Alfa Aesar), pancreatic digest of casein (Difco), potassium dihydrogen phosphate (VWR Chemicals), potassium phosphate dibasic (Thermo Fisher Scientific), sodium chloride (Thermo Fisher Scientific) and yeast extract (Labkem). For the cell viability assays, resazurin (Alfa Aesar), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Merck), and fluorescein diacetate (FDA) (BioChemika) were used. Two stock solutions were prepared with phosphate buffer solution (PBS), one containing 5 mg/ml resazurin and the other containing 5 mg/ml MTT. A stock solution of 5 mg/ml FDA was prepared using acetone (Merck). Additionally, methanol (JMGS) and dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific) were used for other purposes.

4.3.1. Plant material

A previous study (data not published) identified 39 medicinal plants that are traditionally used for treating parasitic infections in traditional Portuguese medicine. From this list, 11 medicinal plants were selected and purchased commercially in dry form from Alfredo Augusto Tavares, Sucessores, Lda (Table 12).

Table 12 – Medicinal plants information.

Scientific Name	Part	Batch	Expiration Date	Origin
Artemisia absinthium L.	Leaf and Stem	04ABS1123J231S	01/26	Bulgaria
Centaurium erythraea Rafn	Leaf and Stem	01FEL273211C	01/26	Albania
Chenopodium ambrosioides L.	Leaf and Stem	05FOR003J231S	05/26	Portugal
Cucurbita pepo L.	Seed	11AB011852001	11/25	No information
Juglans regia L.	Leaf	01NOG587J2218	06/26	No information
Mentha x piperita L.	Leaf	06HOR1106J232S	04/26	Non-EU
Mentha spicata L.	Leaf and Stem	06HOS1182181S	06/26	No information
Olea europaea L.	Leaf	050LI537J221S	05/26	Portugal
Rosmarinus officinalis L.	Leaf	06ALE1172301	06/26	Non-EU
Thymus vulgaris L.	Leaf	05TOM1172202	05/26	Spain
Vitis vinifera L.	Leaf	06VID545J221S	06/26	Portugal

4.3.2. Parasites and culture maintenance

G. lamblia trophozoites (strain Portland-1, ATCC 30888) and *T. vaginalis* trophozoites (strain JH 31A #4, ATCC 30236) were routinely cultivated at 37ºC in modified TYI-S-33 medium (Keister, 1983) and modified TYM Diamond medium (Clark & Diamond, 2002; Diamond, 1957), respectively. Both cell culture media were supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 1% antibiotic-antimycotic 100x solution (Gibco). Additionally, 0.05% of kanamycin (Sigma) was added to TYI-S-33 medium. The maintenance of the cell cultures was performed every 3-4 days with fresh culture media.

Periodic cryopreservation of *G. lamblia* and *T. vaginalis* trophozoites was conducted to preserve the obtained isolates, following modified methods described in both product sheets (American Type Culture Collection, 2022a, 2022b). Briefly, for *G. lamblia* trophozoites, culture tubes containing cells in the exponential growth phase were shaken, and the supernatant was removed. The tubes were subsequently filled with fresh culture medium, immersed in an ice bath for 15 minutes, and shaken periodically to facilitate detachment of trophozoites from the tube walls. Sample aliquots of 1 ml or 2 ml were distributed into cryovials containing 0.5% DMSO and frozen at -80°C. For *T. vaginalis* trophozoites, culture tubes with cells in the

exponential growth phase were kept in vertical position for 10 minutes. Subsequently, 1 ml or 2 ml aliquots were taken from the upper portion of the culture tube and dispensed into cryovials containing 0.5% DMSO before being frozen at -80°C. To reconstitute the samples, the trophozoites were thawed in a water bath at a temperature of 35 to 37°C, followed by resuspension in fresh culture medium.

4.3.3. Preparation of plant extracts

A modification of the methods of Abdeta et al. (2021) and Tahir et al. (1999) was used. First, the dried plant material was ground to a fine powder using a mill (Taurus® Aromatic 150w) for 30 seconds. For the aqueous extract, 10 g of powder from each plant was weighed and macerated with ultrapure water for 3 days, in a ratio of 1:20, at room temperature, using a magnetic stirrer (VWR® Hotplate/Stirrer) at 250 rpm. For the methanolic extracts, the process was similar, with the only difference being the solvent used - 80% (v/v) methanol. After maceration, the mixtures were filtered (VWR® Whatman Grade 1). The methanolic extracts were concentrated in a rotary evaporator (VWR®) at 39ºC, 60 rpm and 900 mbar. The extracts were then lyophilised (Labcon® FreeZone 4.5 l) and stored at -80ºC.

4.3.4. Cell growth evaluation

To determine the most suitable cell concentration for employment in susceptibility assays, an adaptation of the Duarte et al. (2009) method was used. Initially, cell counts were performed on an aliquot retrieved from a 10 ml culture tube containing trophozoites that had been incubated for 72 hours using a Neubauer chamber. The concentrations obtained were $2.5x10^6$ cells/ml for *T. vaginalis* and $1.8x10^6$ cells/ml for *G. lamblia*. Subsequently, seven different cell concentrations were obtained through dilution with cell culture media. All concentrations were added to a 24-well microplate in triplicate and incubated at 37ºC for 24 hours. Following incubation, the parasitic growth of the cultures in each well was evaluated by counting the number of parasites in a Neubauer chamber under an optical microscope at 100x total magnification. In the case of *G. lamblia*, due to the mobility of the parasite when in suspension, the parasites were fixed with 1% paraformaldehyde during sample preparation for counting. Each assay was performed twice.

4.3.5. Comparison of viability assays

To evaluate the most suitable viability assay, a comparative analysis was conducted between a resazurin assay and two alternative assays: the MTT assay for *G. lamblia* and the FDA assay for *T. vaginalis*. Metronidazole working solutions, ranging from 5 µg/ml to 2000 µg/ml, were employed.

For the resazurin assay, a modified version based on the method by Duarte et al. (2009) was used. Briefly, 100 µl of $1x10^6$ log-phase trophozoites were incubated with 100 µl of metronidazole working solutions for 24 hours at 37°C in a 96-well microplate. Following incubation, 20 µl of 1 mg/ml resazurin solution was added to the wells and incubated for 4 hours (for *G. lamblia*) or 30 minutes (for *T. vaginalis*). Fluorescence readings were recorded using the SpectraMax®Mini microplate reader at 535 nm (excitation) and 595 nm (emission).

For the FDA assay, a modified approach based on the method described by Jones & Senft (1985) was employed. Similar to the resazurin assay, trophozoites were incubated with the metronidazole working solutions for 24 hours. Following incubation, 10 µl of 20 µg/ml FDA solution was added to the wells and incubated for 30 minutes. Fluorescence measurements were conducted at 485 nm (excitation) and 535 nm (emission).

For the MTT assay, an adaptation of the method described by Singh et al. (2016) was used. A volume of 100 µl containing $1x10^6$ log-phase trophozoites was incubated with 100 µl of metronidazole working solutions for 24 hours at 37°C in a 96-well microplate. Following incubation, the culture medium was removed, and the cells were rinsed with 100 µl of PBS. Subsequently, 10 µl of MTT stock solution was added, and following a 4-hour incubation period, the supernatant was removed. A solution of 100 µl of DMSO + ethanol (50:50) was added to dissolve the formed crystals, and the absorbance was measured at 570 nm. Each viability assay was performed twice.

4.3.6. Susceptibility assays

The aqueous extracts were solubilized in ultrapure water to obtain a stock solution of 20 mg/ml, while the methanolic extracts were solubilized in DMSO to obtain a stock solution of 400 mg/ml. Plant extract working solutions, ranging from 5 µg/ml to 2000 µg/ml, were prepared by serial dilution with culture media containing 0.5% DMSO. In a 96-well microplate, 100 µl of $1x10^6$ log-phase trophozoites were incubated with 100 µl of plant extract working

solutions in triplicate, for 24 hours at 37°C. A positive control consisting of 100 µl of $1x10^6$ log-phase trophozoites plus 100 µl of culture media containing 0.5% DMSO, as well as a negative control comprising 200 µl of culture media containing 0.5% DMSO, were also added to the microplate. The final DMSO concentration was always less than 1%. Following incubation, cell viability was assessed using the resazurin assay. Each assay was performed twice.

4.3.7. Statistical analysis

The Statistical Software Package for the Social Science (SPSS) Version 29.0.1.0 was used for data analysis. The results of the cell viability assays were compared using the independent samples t-test. If the assumptions for conducting the test were not met, the nonparametric Mann-Whitney U test was employed. Probit analysis was used to calculate the concentration of drug that inhibited trophozoite viability by 50% (IC_{50}). Significance was determined at the 95% confidence level. A p-value less than 0.05 was considered statistically significant.

4.4. Results and discussion

4.4.1. Cell growth evaluation

The selection of an appropriate cell concentration for conducting susceptibility assays is crucial to ensure that cell growth is in the exponential growth phase after the 24-hour incubation period. The determination of the optimal conditions for cell viability assays involving *G. lamblia* and *T. vaginalis* trophozoites was based on the characterization of cell growth, as depicted in Figures 6 and 7.

In *G. lamblia*, concentrations above $1x10^6$ cells/ml displayed comparable growth rates (Figure 6). For *T. vaginalis*, concentrations ranging from $5x10^5$ to $1.5x10^6$ cells/ml exhibited the highest growth rates (Figure 7). As a result, a cell concentration of $1x10^6$ cells/ml was chosen for conducting the susceptibility assays for both parasites. Notably, despite methodological variations, multiple authors have reported the use of this particular cell concentration (Argüello-García et al., 2004; Wachter et al., 2014).

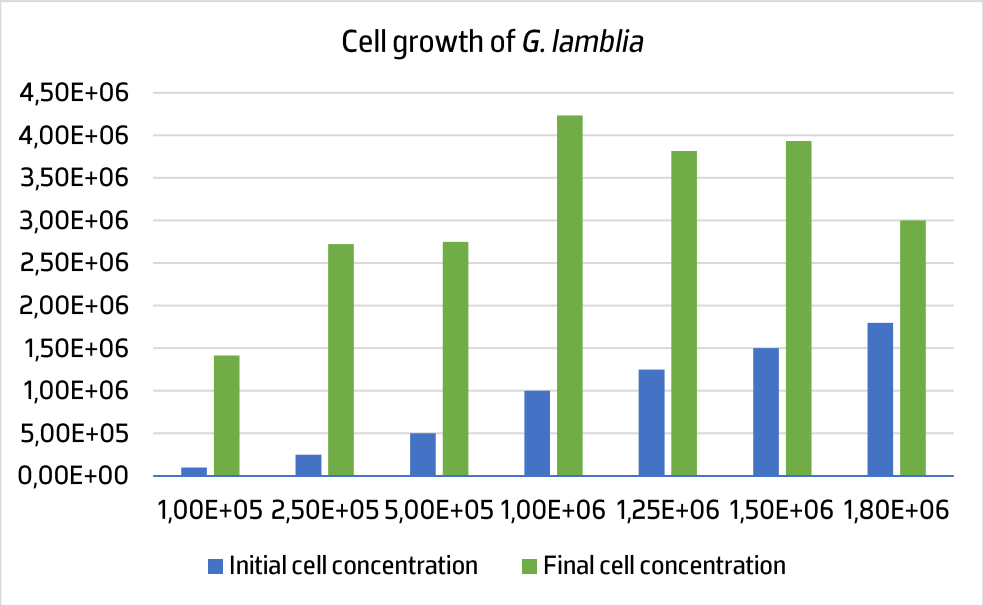

Figure 5 – *G. lamblia* growth assay.

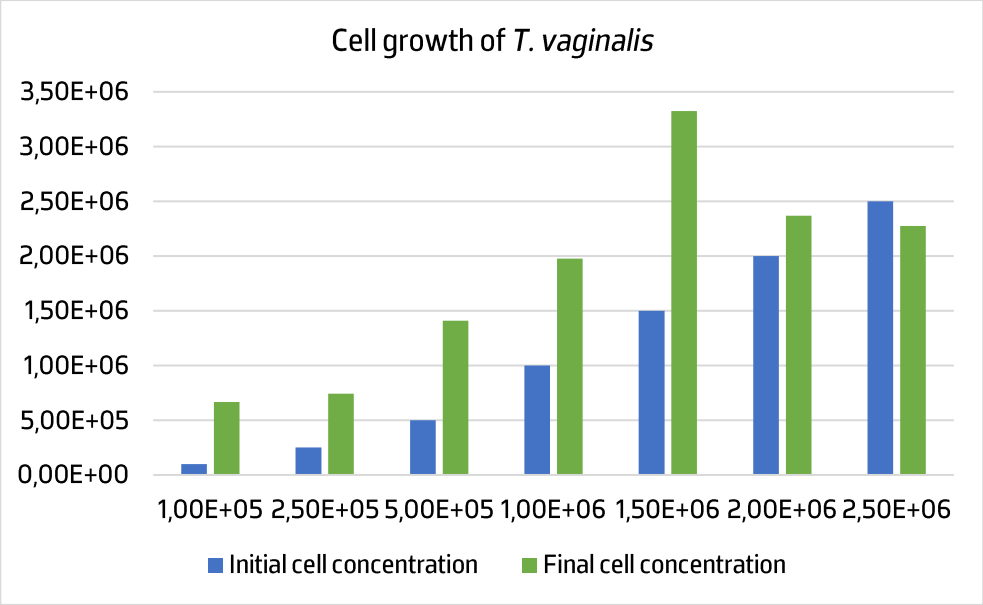

Figure 6 – *T. vaginalis* growth assay.

4.4.2. Comparison of viability assays

The inhibitory effect of metronidazole was evaluated by comparing two methods for each parasite at the endpoint reading. Ideally, the same viability assays should be evaluated for both parasites to ensure uniformity. However, as is widely recognized, the MTT assay requires the removal of cell culture medium, as its components can reduce MTT. While successfully

implemented in previous studies by Sariego et al. (2014) and Singh et al. (2016) for *T. vaginalis*, the step of cell culture media removal presented a challenge due to *T. vaginalis* growth in suspension. Consequently, these challenges led to the substitution of the MTT assay with the FDA assay to assess *T. vaginalis* trophozoite viability.

The obtained IC_{50} values for metronidazole in *G. lamblia* ranged from 12.58 to 13.38 µM, whereas for *T. vaginalis*, the IC_{50} values ranged from 24.37 to 25.45 µM (Table 13). Despite differences in methodology, several authors have reported similar levels of susceptibility. In a study by Cruz et al. (2003), the IC_{50} values of metronidazole in *G. lamblia* varied from 2.38 to 11.50 µM. For *T. vaginalis*, in a study by Seo et al. (2017), the IC_{50} of metronidazole against metronidazole-sensitive strains varied from 11.7 to 22.8 µM, while metronidazole-resistant isolates exhibited higher values compared to sensitive isolates (182.9 to 730.4 µM).

Table 13 – Comparison of viability assays for *G. lamblia* and *T. vaginalis.*

	G. lamblia (IC_{50})		*T. vaginalis* (IC_{50})	
Assay	µg/ml	µM	µg/ml	µM
Resazurin	2.15 ± 0.18	12.58	4.35 ± 0.47	25.45
MTT	2.29 ± 0.24	13.38		
FDA			4.17 ± 0.56	24.37

For *G. lamblia*, the significance of the difference between the means of the methods (resazurin (Group 1) vs. MTT (Group 2)) was assessed using the independent samples t-test. The assumptions of this statistical method, namely, the normality of distributions and the homogeneity of variances, were evaluated using the Shapiro-Wilk test (SWGroup 1: p-value=0.361; SWGroup 2: p-value=0.585) and the Levene test, which is based on the median (p-value=0.,481). As both groups exhibited a normal distribution, the t-student test was used to verify whether there was a statistically significant difference between the means. There was no statistically significant difference between the means of the tested methods, whose test p-value was greater than 0.05. Therefore, the resazurin assay was chosen for evaluating *G. lamblia* trophozoites viability.

For *T. vaginalis*, the significance of the difference between the medians of the methods (resazurin (Group 1) vs. FDA (Group 2)) was evaluated using the nonparametric Mann-Whitney test, as the assumptions for conducting the independent samples t-test were not met, particularly regarding the normality of Group 2. No statistically significant evidence was observed (p-value=0.485), and the test p-value was greater than 0.05. Although the FDA

method resulted in a smaller mean IC_{50}, it had a larger standard deviation. Thus, for standardization, the resazurin assay was also chosen to evaluate the viability of *T. vaginalis* trophozoites.

4.4.3. Plant extracts and susceptibility assays

In the present study, water and methanol were used as solvents to obtain the crude plant extracts. Hydrophilic solvents such as water, methanol, and ethanol are commonly selected for the extraction of polar compounds (Abubakar & Haque, 2020). Initial extraction procedures often involve ethanol or methanol, particularly due to their effectiveness in dissolving aromatic or saturated organic compounds known for their antimicrobial properties (Abubakar & Haque, 2020; Tiwari et al., 2011).

Methanol, which is widely employed in medicinal plant extraction, has the ability to extract a diverse array of phytochemical constituents, including alkaloids, anthocyanins, anthraquinones, flavonoids, phenolic compounds, saponins, tannins, and terpenoids (Dahab et al., 2015; Kebede et al., 2021; Tiwari et al., 2011). These compound are associated with antimicrobial activities, thereby enhancing their potential for therapeutic applications (Das et al., 2010).

Water, known as a universal solvent, is also frequently used in the extraction of plant products with antimicrobial activity. It facilitates the extraction of active components such as anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins (Tiwari et al., 2011). Among these compounds, subclasses such as phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins, have demonstrated potential antimicrobial effects (Das et al., 2010).

The IC_{50} values of the tested aqueous and methanolic extracts of the selected medicinal plants against *G. lamblia* and *T. vaginalis* trophozoite cultures are displayed in Table 14. In terms of growth inhibition, none of the extracts showed effect on either *G. lamblia* or *T. vaginalis*, as the IC_{50} values obtained were higher than the highest concentration tested (2000 µg/ml). All IC_{50} values were classified as low (IC_{50}> 500 µg/ml) according to Moga et al. (2021).

However, in *G. lamblia*, three aqueous extracts (*Artemisia absinthium* L., *Chenopodium ambrosioides* L. and *Cucurbita pepo* L.) at the higher concentrations, 1000 and 2000 µg/ml, exhibited potential in cell proliferation. This observation was qualitatively assessed by visual inspection of the microplate. Upon examination, an increase in the number of cells was

observed in the wells containing higher concentrations of the aqueous extract than in those containing the positive control. This was indicated by the colour change of resorufin from pink to transparent. Additionally, it was confirmed by microscopic examination.

Table 14 – IC_{50} values of the tested aqueous and methanolic extracts in *G. lamblia* and *T. vaginalis.*

Active Principle / Plant	Extract	*G. lamblia* (IC_{50}: µg/ml)	*T. vaginalis* (IC_{50}: µg/ml)
Metronidazole	–	2.15	4.35
Mentha x piperita L.	Aqueous	> 2000	> 2000
	Methanolic		
Mentha spicata L.	Aqueous		
	Methanolic		
Juglans regia L.	Aqueous		
	Methanolic		
Artemisia absinthium L.	Aqueous*		
	Methanolic		
Olea europaea L.	Aqueous		
	Methanolic		
Vitis vinifera L.	Aqueous		
	Methanolic		
Rosmarinus officinalis L.	Aqueous		
	Methanolic		
Chenopodium ambrosioides L.	Aqueous*		
	Methanolic		
Thymus vulgaris L.	Aqueous		
	Methanolic		
Erythraea centaurium L.	Aqueous		
	Methanolic		
Cucurbita pepo L.	Aqueous*		
	Methanolic		

*Potential cell proliferation activity.

Several of the tested medicinal plants have been tested by other researchers for their antiprotozoal activity. However, we decided to evaluate these species because their antiprotozoal properties were demonstrated with different parasites, crude extracts, or plant parts to obtain the extract.

Rosmarinus officinalis L. has been tested against both *G. lamblia* and *T. vaginalis*. Dahab et al. (2015) found that while the aqueous extract of *Rosmarinus officinalis* L. showed no activity against trophozoites of *G. lamblia,* the methanolic extract demonstrated strong activity, with an IC_{50} of 0.689 mg/ml. Similarly, Alkashab et al. (2020) determined that the growth of *T. vaginalis* trophozoites was inhibited by the ethanolic extract (0.1 mg/ml) and the aqueous extract (400 mg/ml) after 1 hour of incubation.

Mentha x piperita L. has also been tested against both *G. lamblia* and *T. vaginalis*. Najumudin et al. (2018) observed a moderate activity of an ethanolic extract against *G. lamblia* trophozoites,

while Vidal et al. (2007) found that all extracts, except for the aqueous extract, reduced the *in vitro* growth of *G. lamblia* trophozoites. Notably, the methanolic extract and the dichloromethane fraction exhibited strong inhibitory activity, with IC_{50} values of 0.8 μg/ml and 0.75 μg/ml, respectively. Aslani et al. (2019) tested a hydroalcoholic extract at a concentration of 5 mg/ml in *T. vaginalis* trophozoites and found complete growth inhibition within 24 hours. In the study by Garza-González et al. (2017), a 300 μg/ml methanolic extract of *Thymus vulgaris L.* showed antiprotozoal activity against *G. lamblia* (IC_{50} = 86.41 ± 2.9 μg/ml) and *T. vaginalis* (IC_{50} = 115.41 ± 2.29 μg/ml).

Calzada et al. (2006) studied the effects of methanolic extracts of *Artemisia absinthium* L., *Chenopodium ambrosioides* L. and *Thymus vulgaris* L. on *G. lamblia,* obtaining IC_{50} values of 135.4 μg/ml; 116.1 μg/ml and 68.7 μg/ml, respectively. Furthermore, Calzada et al. (2007) studied the effects of methanolic extracts of the same plants on *T. vaginalis,* obtaining IC_{50} values of 708.6 μg/ml; 996.7 μg/ml and 126.4 μg/ml, respectively.

Lastly, Neiva et al. (2014) tested two ethanolic extracts of *Chenopodium ambrosioides* L. prepared through maceration and percolation, which demonstrated high giardicidal activity, with IC_{50} values of 214.16 ± 5.02 μg/ml and 198.18 ± 4.28 μg/ml, respectively.

Across these studies, notably, the aqueous extracts rarely exhibited any effect. A plausible explanation for this diminished activity may be attributed to the action of the enzyme polyphenol oxidase, which degrades polyphenols in water extracts while remaining inactive in methanol and ethanol extracts (Tiwari et al., 2011). The variation observed in trophozoite growth inhibition among the cited studies and the present investigation may be attributed to multiple variables that interfere with the synthesis of secondary metabolites of the plant material. These variables include, but are not limited to, geographical location and time of plant collection, collection and botanical identification process of plant material, use of fresh or dried plant material, plant age, drying process, choice of solvent and its concentration, extraction method employed, strain of parasite, and the experimental model employed for testing purposes (microscale and viability assay used). Each of these variables has the potential to significantly impact the bioactivity and efficacy of the extracted compounds, thereby inducing different outcomes across studies.

4.5. Conclusion

The aim of the present study was to assess the antiparasitic potential of 11 medicinal plants, comprising 22 extracts, which are traditionally used in Portuguese traditional medicine to treat parasitic diseases, against *G. lamblia* and *T. vaginalis* trophozoites. Our findings indicate that none of the tested plant extracts exhibited significant antiparasitic activity, as evidenced by the IC_{50} surpassing the higher concentration tested (2000 μg/ml). However, the aqueous extracts of *Artemisia absinthium* L., *Chenopodium ambrosioides* L. and *Cucurbita pepo* L. demonstrated promising potential in promoting cell proliferation of *G. lamblia* at higher concentrations. For future investigations, it would be interesting to evaluate the same medicinal plants against both *G. lamblia* and *T. vaginalis*, as well as other parasites, such as helminths, by employing different extraction processes and alternative solvents. This broader approach may yield valuable insights into the potential antiparasitic properties of these medicinal plants and facilitate the development of effective treatments for parasitic infections. Additionally, it would be beneficial to explore whether the observed potential of cell proliferation by the mentioned aqueous extracts in *G. lamblia* is indeed attributable to their properties or if it could result from protective effects against induced toxicity. These investigations would provide valuable insights into the mechanisms underlying the observed effects and further elucidate the potential therapeutic applications of these medicinal plants in combating parasitic infections.

4.6. References

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5. Conclusion

Infectious diseases caused by protozoa remain a significant public health problem, and more pharmacological options are needed to combat these diseases. *G. lamblia* is one of the most common waterborne parasites that infects humans, while *T. vaginalis* is the cause of the most prevalent curable parasitic sexually transmitted disease.

In the first chapter, a variety of methods used throughout the *G. lamblia* life cycle were identified. The isolation and purification step demonstrated the most variability, with density gradients commonly used for faecal samples and immunomagnetic separation (IMS) for water samples. While other stages showed more uniform methods, standardization across all methods remains a challenge. Further validation for clinical use and ongoing research is crucial to improve these techniques and establish best practices.

In the second chapter, a wide variety of viability assessment methods for both *G. lamblia* and *T. vaginalis* were also identified. Common assays included cell count, cell motility, resazurin, SYBR Green I, and trypan blue, with cell counting being the most cited. However, this method is subjective, insensitive, laborious, and time-consuming. Resazurin and SYBR Green I demonstrated high accuracy but required modifications to the cell culture medium. Understanding the strengths and weaknesses of each method is vital for informed decision-making in research and diagnostic testing. Further standardization of viability assays is essential to advance the understanding of drug susceptibility and effective treatment development.

In the third and final chapter, the antiparasitic potential of 22 plant extracts against *G. lamblia* and *T. vaginalis* trophozoites was assessed. While none of the extracts exhibited significant antiparasitic activity, the aqueous extracts of *Artemisia absinthium* L., *Chenopodium ambrosioides* L., and *Cucurbita pepo* L. demonstrated promising potential for promoting cell proliferation at the higher concentrations. Future research should explore whether this effect is due to protective effects against induced toxicity or inherent proliferative properties. Different extraction processes and solvents should also be investigated, as some plants have previously exhibited antiparasitic potential in other studies.

Several limitations emerged. First, the variability in the methodologies used across different stages of the *G. lamblia* life cycle may affect the reproducibility of the results. Despite efforts to standardize techniques, some methods require further validation, which could influence the accuracy and reliability of the findings. Despite efforts to standardize methodologies, there

may still be inconsistencies in how techniques are applied, potentially affecting the reliability of the results. Second, the viability assessment methods, while comprehensive, may still suffer from subjectivity and variability, particularly with the cell counting method. This could impact the consistency of the viability results. Third, the plant extracts tested did not exhibit significant antiparasitic activity, which may be due to the specific extraction methods and solvents used. The choice of extraction method (aqueous/methanolic) may not have been optimal for all plant compounds. Different extraction processes might yield more potent antiparasitic compounds. Moreover, the tested plants were acquired commercially in dry form, and other studies collected the plants and prepared them for further testing because the environmental conditions under which the plants were grown, harvested, and stored might affect their chemical composition and, consequently, their antiparasitic activity.

The findings from all three articles contribute to advancing research methodologies for *G. lamblia* and *T. vaginalis*. Improved isolation and viability assessment techniques can lead to a better understanding and control of these infections, with substantial potential clinical and biological applications. Standardized protocols for isolation and viability assessment can enhance diagnostic accuracy, which is crucial for reproducibility and cross-study comparisons of results.

Given the limited data on trichomoniasis and its potential significance, mandatory reporting of *T. vaginalis* infections should be considered. Future research should focus on refining and validating isolation and viability assessment techniques, focusing on cost-effective, rapid, and accurate methods that benefit both research and clinical diagnostics.

The exploration of ethnobotanical resources remains essential. Identifying and testing new plant-based compounds with antiparasitic properties could lead to novel treatments, addressing the growing issue of drug resistance. These advancements underscore the importance of integrating improved diagnostic methodologies and novel treatment options to improve global health outcomes.

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