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Original Article

A comprehensive analysis of viability assays for *Giardia lamblia* and *Trichomonas vaginalis* trophozoites: a systematic review

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Abstract

Introduction: *Giardia lamblia* and *Trichomonas vaginalis* are flagellated protozoan parasites that often cause asymptomatic infections but may lead to gastrointestinal or genitourinary symptoms. Improved treatment options are needed due to emerging resistance. 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 aimed 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 for *G. lamblia* and *T. vaginalis*.

Methodology: 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").

Results: The search identified 32 experimental studies with diverse viability assays. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, adherence inhibition assay, and [3H]-thymidine incorporation assay were prominent for *G. lamblia*. The trypan blue assay, motility assessment, and resazurin assay were frequently used for *T. vaginalis*. 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.

Conclusions: The fluorometric resazurin assay has emerged as a suitable choice for standardization in both parasites, offering cost-effectiveness, reliability, and ease of use.

Key words: cell viability; giardiasis; parasitic diseases; parasite viability; protozoan parasites; trichomoniasis.

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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 diarrhea, 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 feces [1,2]. In 2019, 18,004 confirmed cases of giardiasis were reported in the European Union and European Economic Area [1].

Trichomonas vaginalis, another flagellated protozoan parasite, is the etiological agent of trichomoniasis which is a genitourinary parasitic infection responsible for an estimated 156 million new cases annually [3,4]. This disease is generally asymptomatic in men; however, it may manifest in women through symptoms such as vaginal discharge and vulvar pruritus [3,5]. Trichomoniasis is primarily sexually transmitted, with the protozoan existing predominantly in the trophozoite form, where the flagella are externalized [3,6]. While trichomonads do not present a true cyst form, endoflagellar forms have been observed [6].

Pharmacological treatment for both infections relies primarily on metronidazole (a 5-nitroimidazole) [6]. In the case of giardiasis, albendazole (a benzimidazole) is also frequently employed as a therapeutic agent [6,7]. 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 [2,6,7]. Furthermore, treatment

failure and reinfection are not uncommon [8]. 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 [9,10].

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 [11]. In addition, *in vitro* culturing of trophozoites remains the standard laboratory tool for drug discovery, resistance monitoring and fundamental research [10]. 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 [9,12,13]. 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 the 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 aimed to propose a standardized viability assay for susceptibility testing of *G. lamblia* and *T. vaginalis*.

Methodology

This systematic review was conducted according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA 2020) guidelines [14]. The SPIDER tool adapted from Cooke *et al*. [15] was employed to formulate the following research question: "What methodologies are most commonly employed in *in vitro* studies for assessing the anti-giardial and anti-trichomonal activity of compounds with potential therapeutic activity?" (Table 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 case of the ScienceDirect database, the search was based on title, abstract, or author-specified keywords. The available MeSH terms were used in MEDLINE. The databases were accessed on 21 August 2024.

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.

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*. [16]. 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" and by quality into a scale: 1–2

Table 1. Description of the SPIDER tool adapted from [15].

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

Table 2. Quality assessment criteria used for the evaluation of the articles (adapted from [16]).

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?			

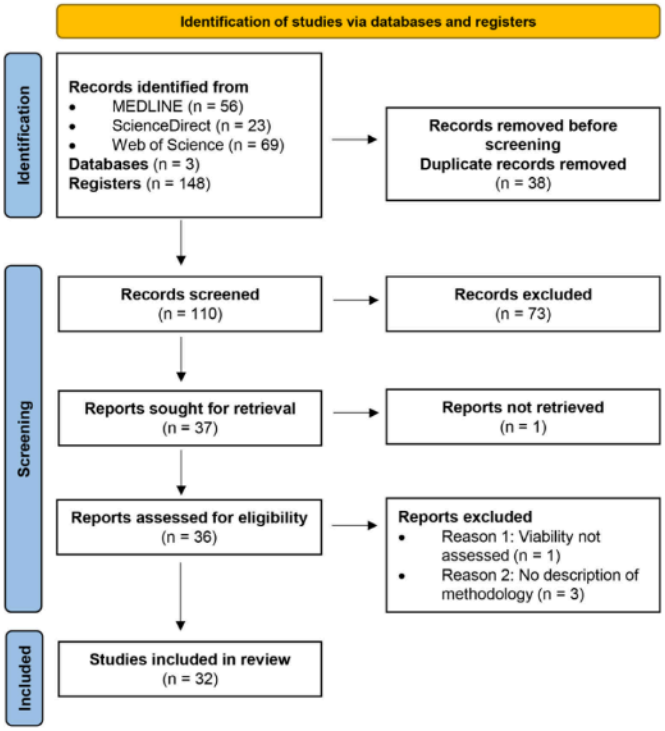
unsatisfactory, 3–4 satisfactory, 5–6 good, and 7–8 excellent.

Results

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 1). These records were downloaded from each respective database and then integrated into the Rayyan platform [17]. 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. When 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 1 illustrates and summarizes the complete study selection process.

Figure 1. Selection procedure, adapted from the PRISMA 2020 statement [14].



Quality assessment

In order 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 2). 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.

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. The advantages and disadvantages were extracted exclusively from the original studies included in this systematic review. No additional data from external sources were added, in order to preserve methodological rigor and avoid interpretation bias. Therefore, for some methods, advantages, or disadvantages may not be listed in Tables 3 and 4.

Viability assays for G. lamblia

A total of 21 viability assays were identified for *G. lamblia* (Table 3). The category with the highest number of assays was the colorimetric group, which included 8 assays; followed by the morphophysiological group, which comprised 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 [3H]-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 [18].

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 [19]. Adherence serves as a crucial viability indicator, with adherence levels quantified by enumerating the adhered trophozoites or measuring the optical density of stained trophozoites [20].

The [3H]-thymidine assay involves labelling cells with radioactive [3H]-thymidine, which is then incorporated into their deoxyribonucleic acid (DNA). The amount of [3H]-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 [3H]-thymidine into their DNA than non-dividing or declining cells [21].

Viability assays for T. vaginalis

A total of 8 viability assays were identified for *T. vaginalis* (Table 4). 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 [22–24]. The motility assay relies on visual examination of cell motility using an inverted microscope [25]. Typically, all motile cells, including stationary ones displaying movement of flagella and/or undulating membranes, are counted [26,27]. Some authors solely observe motility, while others quantify motile cells using a hemocytometer [25,27]. The resazurin assay involves the conversion of the non-fluorescent blue dye, resazurin, to pink fluorescent resorufin by the mitochondrial enzymes of viable cells [22–28]. 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 color change indicating the presence or absence of viable cells [22,28].

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

Article	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Overall
Afzan *et al.* [52]	✓	✓	✓	✓	✓	✓	✗	✗	
Aldrete *et al.* [35]	✓	✓	✓	✓	✓	✓	✗	✓	
Argüello-García *et al.* [40]	✓	✓	✓	✓	✓	✓	✓	✓	
Barbosa *et al.* [43]	✓	✓	✓	✓	✓	✓	✗	✓	
Bénéré *et al.* [33]	✓	✓	✓	✓	✓	✓	✓	✓	
Boreham *et al.* [42]	✓	✓	✓	✓	✗	✓	✓	✓	
Bromke *et al.* [26]	✓	✓	✓	✓	✗	✓	✗	✓	
Chaudhari and Singh [27]	✓	✓	✓	✗	✗	✓	✗	✓	
Chen *et al.* [44]	✓	✓	✓	✓	✓	✓	✗	?	
Chen *et al.* [56]	✓	✓	✓	✓	✓	✓	✗	✓	
Crouch *et al.* [46]	✓	✓	✓	✓	✓	✓	✗	✗	
Cruz *et al.* [47]	✓	✓	✓	✓	✓	✓	✗	✓	
Cruz *et al.* [48]	✓	✓	✓	✓	✓	✓	✗	✗	
Downey *et al.* [45]	✓	✓	✓	✓	✓	✓	✗	✓	
Escribano *et al.* [34]	✓	✓	✓	✓	✓	✓	✓	✓	
Gadelha *et al.* [39]	✓	✓	✓	✓	✓	✓	✗	✗	
Gordts *et al.* [50]	✓	✓	✓	✓	✗	✓	✗	✓	
Hezarjaribi *et al.* [53]	✓	?	✓	✓	✗	✓	✗	✓	
Hounkong *et al.* [31]	✓	✓	✓	✓	✓	✓	✗	✓	
Kang *et al.* [21]	✓	✓	✓	✓	?	✓	✓	✓	
Karami *et al.* [58]	✓	✓	✓	✓	✓	✓	✓	✓	
Karami *et al.* [59]	✓	✓	✓	✓	✓	?	✗	✓	
Lara-Díaz *et al.* [49]	✓	?	✓	?	✓	?	✗	✓	
Meri *et al.* [25]	✓	✓	✓	✓	?	✓	✗	✓	
Naidoo *et al.* [51]	✓	?	✓	✓	✗	✓	✓	✓	
Osmari *et al.* [54]	✓	?	✓	✓	✓	✓	✗	?	
Özel *et al.* [60]	✓	✓	✓	✓	?	✓	✗	✓	
Ponce-Macotela *et al.* [41]	✓	✓	?	✓	✓	✓	✗	✓	
Smith and Domenico [57]	✓	✓	✓	✓	✗	?	✗	✓	
Tiwari *et al.* [55]	✓	✓	?	✓	?	✓	✗	✓	
Wright *et al.* [32]	✓	✓	✓	✓	?	✗	✗	✗	
Yadegari *et al.* [38]	✓	✓	✓	?	✓	✗	✗	✓	

Caption

✓ Yes
? Not applicable/ not sure
✗ No

Excellent
Good
Satisfactory
Unsatisfactory

Table 3. Assays for viability assessment of *Giardia lamblia* trophozoites.

Type of assay	Assay name	Parameter	Cited by[1]	Advantages	Disadvantages	References[2]
Colorimetric	Crystal violet	IC_{50}	[31]	• Shows a more satisfactory OD reading than eosin	• Has tendency to stain the tissue culture plate	[21,31–33]
	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}	[21,38]	–	• Less satisfactory OD reading than crystal violet and methylene blue	
	MTS/PMS	IC_{50}	[39]	–	• Removal of culture medium is necessary • Requires multiple wash steps	
	MTT	IC_{50}	[33,40, 41]	–	• 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}	[21]	• Simple • Determined directly by the change in absorbance • Both continuous and stopped assays are feasible • Not dependent upon a coupled enzyme system	–	
	Trypan blue	ID_{50}	[33,42]	• Simple • Widely used	• Laborious • Time-consuming • Requires a microscopic examination to count cells with a haemocytometer	
	XTT	IC_{50}	[32,33]	• 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	
Fluorometric	Resazurin or Alamar blue	IC_{50}	[33]	• 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	[31,33,40,43–45]
	FDA-PI	IC_{50}	[40]	• Fast • Cost-effective • Efficient • Can be evaluated by flow cytometry	• Requires multiple wash steps • Requires a microscopic examination to count cells	
	PI	IC_{50}	[43]	• 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}	[45]	• 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	
Morpho-physiological	Adherence Inhibition	IC_{50}	[43,46–48]	• 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	[21,31,40, 46,47]
	Growth Inhibition or Multiplication Method	IC_{50}	[46,47]	• 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 hemocytometer	
	Cell Count	IC_{50} %GI	[31,33, 49]	-	• Laborious • Time-consuming • Subjective • Difficult to assay for several compounds at the same time	
	Cell Morphology	IC_{50}	[40]	• 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 hemocytometer • Subjective • Difficult to assay for several compounds at the same time	
	Cell Motility	MIC	[42]	-	• Unreliable • Low sensitivity • Lacks standardization • Does not consider that organisms may be reproductively non-viable while still showing flagellar activity	
Luminescence	ATP Content	IC_{50}	[44]	• Nonbiased • Homogeneous format (amenable to high-throughput screens) • Reproductible • Dynamic follow-up experiments remain possible	-	[44]
Micro-biological	MMSM or CGSSM	MIC	[50]	-	• Impracticable • Requires large volumes of culture medium • Expense of materials • Problems of replication • Plating efficiency of only 25%	[40,46,50]

Type of assay	Assay name	Parameter	Cited by[1]	Advantages	Disadvantages	References[2]
					• Requires a microscopic examination to count cells with a hemocytometer	
	SCLM	IC_{50}	[40]	• Highly sensitive method for assessing the effects of 5-nitroimidazoles and benzimidazoles	• Indirect • Time-consuming • Requires a microscopic examination to count cells with a hemocytometer • Efficiently replaced by other viability assays	
Radioisotopic	[^{3}H]-thymidine	ID_{50} IC_{50}	[40,42,45]	• 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	[21,31,42,44]

[1]Cited by refers to the studies included in this systematic review that applied the respective viability assay in their methodologies. [2]References refer to the studies that reported advantages and/or disadvantages of each assay. These studies may or may not correspond to those listed under cited by. 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.

Table 4. Assays for viability assessment of *T. vaginalis* trophozoites.

Type of assay	Assay name	Parameter	Cited by[1]	Advantages	Disadvantages	References[2]
Colorimetric	INT	-	[51]	• Allows qualitative viability assessment	• Time-consuming (needs six hours to act)	[35,51–55]
	Modified field stain	-	[52]	• Provides visualization of internal morphological changes	• Requires a microscopic examination to count cells with a hemocytometer	
	Trypan blue	MIC IC_{50}	[52–55]	• 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	[34, 35]	• 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	[34,35,56]
	SYBR green I	IC_{50}	[56]	• 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	
Microbiological	Disk broth method	MIC	[57]	• Simple • Reliable for detection of metronidazole-resistant strains	• Requires a microscopic examination to count cells with a hemocytometer	[57]
Morpho-physiological	Cell count	MLC IC_{50} %GI	[49,58,59]	–	• Laborious • Time-consuming • Low sensitivity • Subjective (dependent on the experience of the observer)	[25–27,34,35]
	Cell motility	MLC MIC	[25–27,60]	–	• Requires a microscopic examination to count cells with a haemocytometer • Laborious • Time-consuming	

[1]Cited by refers to the studies included in this systematic review that applied the respective viability assay in their methodologies. [2]References refer to the studies that reported advantages and/or disadvantages of each assay. These studies may or may not correspond to those listed under 'cited by'. DNA: deoxyribonucleic acid; EC50: 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.

Viability assays common to both parasites

Only 5 viability assays were common to both *G. lamblia* and *T. vaginalis*: a colorimetric approach (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 hemocytometer, followed by microscopic examination. The incorporation of specific dyes is customary to distinguish between viable and non-viable cells [29]. Alternatively, an automated cell counting system can be employed, offering benefits such as reduced analyst-dependent variability and shorter analytical times when compared to traditional manual methods [30].

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. The 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 [23]. As expected, 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 [23]. 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 [22]. This underscores the necessity to standardize the parameter for evaluating cell viability.

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 [31]. 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 interference with its metabolic reduction, potentially leading to inaccurate measurements [32]. Moreover, numerous colorimetric, morphophysiological, and microbiological methods require a subsequent microscopic examination for cell counting using a hemocytometer, 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 [33,34]. 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 [33,34]. On the other hand, some researchers opt not to alter or remove the culture medium [35]. These divergent methodological approaches underscore the imperative need for a standardized protocol to ensure consistency of results, 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 [22]. Furthermore, while some studies express a preference for certain viability assessment methods over others, this analysis reveals that the choice of method was often influenced by factors such as availability of resources, 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 assessment method.

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 [36]. The inoculum should contain 104 to 106 trophozoites per well and the incubation time should be 1 hour for *T. vaginalis* and 2–4 hours for *G. lamblia* at 37 ºC under low oxygen conditions [33,34,37]. Background (medium only), vehicle (DMSO ($\leq$ 0.1%) or other), and no vehicle (parasites and media) should be included as control wells in each plate [37]. 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 [34,36]. This method is a 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 [33,34]. 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*. The MTT assay presents a valid alternative for assessing the viability of both parasites in the absence of a fluorimeter [13]. 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 [18].

Conclusions

This 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.

The commonly used methods for *G. lamblia* include the MTT assay, adherence inhibition assay, and [3H]-thymidine incorporation assay. The trypan blue exclusion assay, motility assessment, and resazurin assay were commonly used for *T. vaginalis*.

The analysis also highlights the importance of minimizing subjectivity in qualitative assays, including appropriate controls such as untreated cells and solvent controls, and addressing inconsistencies in protocol execution. Among the methods reviewed, the fluorometric resazurin assay emerged as the most reliable and cost-effective choice for assessing trophozoite viability, with the MTT assay serving as a practical alternative in resource-limited settings.

Further research and standardization of viability assessment methods are essential for the understanding of drug susceptibility and the development of effective treatment strategies for these parasitic infections.

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Conflict of interest

No conflict of interest is declared.

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