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Natural treatments for the control of *Giardia duodenalis* infection: *focus* on the potential use of *Tabebuia avellanedae*

Trattamenti naturali per il controllo dell'infezione da *Giardia duodenalis*: *focus* sulle potenzialità di utilizzo di *Tabebuia avellanedae*

Ph.D. CANDIDATE
Giulia Rigamonti
Giulia Rigamonti

SUPERVISOR:
Prof. Fabrizia Veronesi

Ph.D. COORDINATOR:
Prof. Beniamino Terzo Cenci Goga

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Summary

Giardia duodenalis is a protozoan parasite responsible for giardiasis, a disease characterized by acute diarrhea that can progress to a chronic form, though most infections are asymptomatic. The parasite has a simple and direct life cycle consisting of two stages: the trophozoite, which causes symptoms, and the cyst, the infective form released in the environment. Giardiasis is widespread globally, with 250-300 million symptomatic human cases annually, especially impacting vulnerable populations in developing countries where poor hygiene and socioeconomic conditions prevail. In addition to human health, *G. duodenalis* also affects pets and livestock, making it a public health issue not only in the human field, but also in veterinary medicine. Treatment of giardiasis typically involves drugs like metronidazole but concerns about side effects and emerging drug resistance highlight the need for alternative therapies. Furthermore, the potential carcinogenic risks of metronidazole have prompted interest in natural remedies. Medicinal plants and natural compounds have shown promise in the treatment of giardiasis, with several ethnobotanical studies identifying potential candidates. The present Thesis investigates the efficacy of various natural extracts, including *Morinda citrifolia*, *Panax ginseng*, *Tabebuia avellanedae*, and *Zingiber officinale*, as well as the bioactive compounds β-lapachone and 6-gingerol, against *G. duodenalis* trophozoites *in vitro*. Among these, *T. avellanedae* demonstrated significant efficacy with its bioactive compound, β-lapachone, exhibiting lower IC_{50} values than metronidazole. Subsequent toxicity testing in cell lines and intestinal organoid monolayers confirmed their safety, positioning *T. avellanedae* as a promising candidate for further clinical development as an alternative treatment for Giardia.

Introduction

Giardia duodenalis (syn. *Giardia intestinalis* or *Giardia lamblia*) is a protozoan parasite that infects the upper intestinal tract of both humans and animals [1]. Giardiasis, the disease it causes, typically presents as acute diarrhea, which can progress to a chronic form, although most infections remain asymptomatic [2]. The life cycle of *G. duodenalis* is simple and direct, comprising two stages: the trophozoite, an actively replicating stage responsible for the symptoms, and the cyst, a resistant environmental stage that is shed in stool and acts as the infective form. *Giardia duodenalis* is widely distributed globally, with 250-300 million symptomatic human infections reported annually [3]. Vulnerable populations, such as children, the elderly, and immunocompromised individuals, are particularly affected by the disease [4]. Its impact is most severe in developing countries, where it is closely linked to poor socioeconomic and hygienic conditions [1,3]. In 2004, the World Health Organization (WHO) classified giardiasis as a neglected disease of poverty, recognizing its role in hindering development and socioeconomic progress [5].

As a zoonotic disease, giardiasis also affects livestock, leading to significant economic losses due to poor growth, weight loss, and reduced productivity [6], and it has public health implications, particularly in pets, with symptomatic cases often seen in puppies and kittens [7]. Thus, giardiasis has substantial consequences for both human and animal populations.

Several medications are recommended for the treatment of giardiasis in humans, including metronidazole, tinidazole, quinacrine, albendazole, furazolidone, and paromomycin, while metronidazole and fenbendazole are used for animals [8,9]. These chemotherapeutic agents may have adverse side effects, including gastrointestinal problems, nausea, vomiting, metallic taste, headache, leukopenia, mutagenic and carcinogenic effects [10].

However, metronidazole has been widely used for years to treat *G. duodenalis* and potential resistance to the drug is becoming of significant concern [11,12]. To date, no resistant strains have been isolated from patients in clinical settings but in recent years, treatment failures with metronidazole have been reported in 10-20% of human cases [13,14]. Although no cases of drug resistance have been reported in veterinary field, a recent study found metronidazole ineffective against *G. duodenalis* in dogs [15].

In light of the risks linked to chemical drugs, the World Health Organization (WHO) has proposed the use of medicinal plants and other natural products, noting that approximately 80% of the global population relies on traditional remedies for healthcare [16,17]. Ethnobotanical and ethnopharmacological studies have identified a variety of natural products used in the treatment of giardiasis, with varying degrees of effectiveness [18]. Plant-based natural treatments have shown significant potential due to their documented antiprotozoal properties in various studies.

The present Thesis provides a general overview of *G. duodenalis* and the natural treatments tested *in vitro*, *in vivo*, and in clinical trials. In the experimental section, a series of natural extracts (*Morinda citrifolia*, *Panax ginseng*, *Tabebuia avellanedae*, and *Zingiber officinale*) as well as two isolated bioactive compounds (6-gingerol from *Zingiber officinale* and β-lapachone from *Tabebuia avellanedae*), were tested *in vitro* against *G. duodenalis* trophozoites (Assemblage AI, clone WBC6, and Assemblage B, clone GS/M) to determine their efficacy. Among the natural compounds tested, *T. avellanedae* and its bioactive compound β-lapachone was identified as effective against trophozoites *in vitro*. Consequently, we decided to focus on this natural product and further test it both on the same assemblages in a different laboratory (Robert Koch Institute, Berlin) and on other assemblage clone (Assemblage B, clone P424/A5). Additionally, since the compounds needed to be not only effective but also safe, we first evaluated their toxicity by testing it against cell lines (Caco-2 and MDCK). Later, we tested them on monolayers derived from intestinal organoids, which better mimic the intestinal epithelium compared to classical cell lines.

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Chapter 1: *Giardia duodenalis*

1.1 Classification, biology and life cycle

1.1.1 Classification

Giardia is a protozoan parasite belonging to the phylum Metamonada, class Fornicata, order Diplomonadida, family Hexamitidae [1]. Species of the genus *Giardia* can infect a variety of hosts, including mammals, amphibians, and birds. To date, seven species have been recognized as valid: *G. agilis* in amphibians, *G. ardeae* and *G. psittaci* in birds, *G. microti* and *G. muris* in rodents, *G. peramelis* in small marsupial, and *G. duodenalis* in mammals, including humans [2] (**Table 1**).

Species name	Host
G. agilis	Amphibians
G. ardeae	Birds
G. duodenalis	Mammals, including humans
G. microti	Voles and muskrats
G. muris	Rodents
G. peramelis	Small marsupial
G. psittaci	Birds

Table 1. *Giardia* species.

Despite this protozoan was first described by Antonie van Leuuwenhoek in 1681, who observed it in his own diarrheal stools using a microscope [3], the first microscopic illustrations of the parasite's morphological characteristics were defined by Vilém Dušan Lambl in 1859, examining a child's stool (**Figure 1**). The observed microorganism was originally named by Lambl as *Cercomonas intestinalis*, however in 1888 Raphael Anatole Émile Blanchard revised the name to *Lamblia intestinalis*. Finally, in 1915, Charles Wardel Stiles renamed the specie *Giardia lamblia* to honor the contributions of Alfred Giard and Lambl [4].

Figure 1. First microscopic drawing of the parasite *Giardia*, detail [4].

Up to date *Giardia duodenalis* (synonyms *G. lamblia* and *G. intestinalis*) is the only species responsible for infections in humans, and due to its significance as a pathogen in both humans and animals, most of the research has concentrated on its characterizing. Studies of isoenzyme [2] and nucleic acid polymorphisms [2,5] have revealed distinct groups of genetically related strains, known as Assemblages, which are often associated with specific host species. Consequently, eight Assemblages have been identified: Assemblages A and B have a wide host range, affecting both humans and various mammalian species, categorize them zoonotic. Assemblages C and D are found in dogs and wild canids, Assemblage E in artiodactyls, Assemblage F in cats, Assemblage G in rodents, and Assemblage H in pinnipeds [2] (**Figure 2**). A recent comprehensive analysis of 8,409 gene sequences across three loci has confirmed specific host associations for various *Giardia* Assemblages and sub-Assemblages, with support for recognizing Assemblages AI and AII as distinct species. The study recommends aligning Assemblages with historical species designations based on host associations, while proposing new names where descriptions are currently absent. Key taxonomic revisions include the reclassification of *G. duodenalis* Assemblages AI and AII as *G. duodenalis* and *G. intestinalis*, respectively. Additionally, Assemblages B, C, E, F, and G have been reclassified as *G. enterica*, *G. canis*, *G. bovis*, *G. cati*, and *G. simoni*. Proposed new names also include *G. lupus* for the canid-associated Assemblage D, with *G. cervus* and *G. pinnipedis* suggested for cervid- and pinniped-associated types, respectively. This refined taxonomy enhances

the clarity of host-specific *Giardia* infections, allowing for more precise species identification and classification [2].

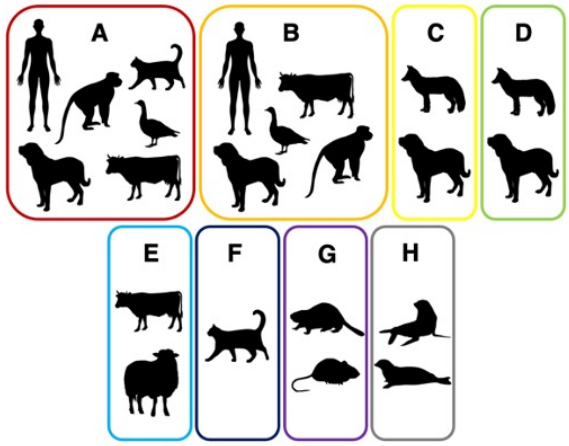

Figure 2. *Giardia duodenalis* assemblages and their potential host.

1.1.2 Biology

Giardia duodenalis exhibits two forms: i) trophozoite, the vegetative/active form, which is characterized by its ability to adhere to host cells and proliferate in the host's small intestine and ii) the cyst, which is dormant, immobile, and serves as the infective form (**Figure 3**) [6].

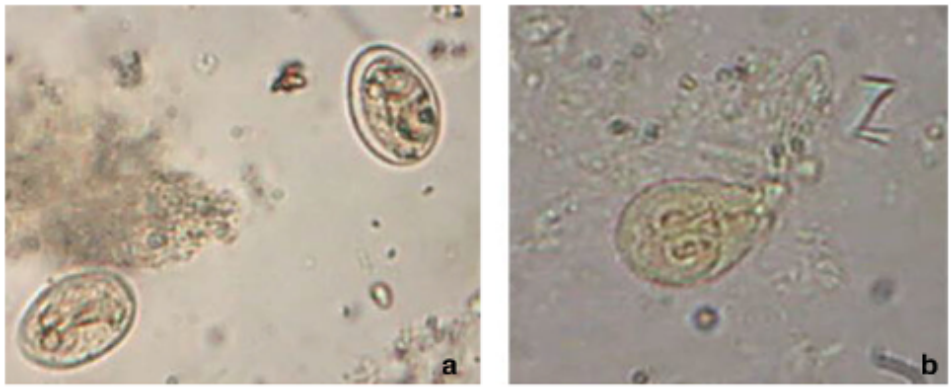

Figure 3. *Giardia intestinalis* in stool sample **a.** cysts **b.** trophozoites [7].

The cysts (5-10 μm) possess a two-layered wall; the outer layer is filamentous, with filaments measuring 7 to 20 nm in length, and contains N-acetylgalactosamine as the primary sugar [8]. Unlike trophozoites, which

have two nuclei, cysts contain four nuclei; furthermore, their metabolic activity is reduced to only 10%-20% of that of trophozoites [9], enabling them to endure in the environment for extended periods, especially in cooler, moist areas [8,10]. The trophozoites exhibit a pear-like shape (12-15 μm in length; 8-10 μm in width); they present four pairs of flagella, a convex dorsal surface with two symmetrically large nuclei in the anterior half, and a concave ventral surface, by a lateral crest and a flange, enabling attachment to the intestinal epithelium [8]. The trophozoites are adapted for survival in the small intestine without invading tissues, with the cytoskeleton (comprising the ventral disk, median body, and eight flagella, each anchored by its respective basal body) playing a crucial role in this adaptation. The fundamental structure of the cytoskeleton is composed of microtubules derived from α- and β-tubulin proteins [8,11,12]. The ventral disk consists of a spiral arrangement of microtubules and associated sheets known as microribbons [8]; it contains numerous disk-associated proteins, including ankyrins (originally termed α-giardins) [13], which contribute to the structural rigidity of the disk's concavity [8,14]. The disk can contract during attachment, utilizing the lateral crest to facilitate initial adhesion and maintain attachment [8,15–17]. The flagella display the typical eukaryotic 9 + 2 microtubule arrangement and are categorized as anterior, posterolateral, ventral, and caudal based on their emergence from the basal bodies situated between the nuclei [14]. While the flagella enable motility in trophozoites, their exact role in adhesion is still a topic of debate [8]. The beating of the ventral flagella coincides with attachment to the intestinal epithelium, supporting a model in which these flagella generate hydrodynamic forces that enable the ventral disk to create suction [18]. A more recent study suggests that the ventral flagella may contribute to suction by pressing the ventral disk against the intestinal epithelium and removing fluid beneath the disk, thus facilitating initial adhesion [19]. The median body is distinctive to *Giardia* species, but also serves as a distinguishing feature among different species. For example, the median body of *G. duodenalis* has been described as resembling a “crooked smile” [6]. Although its precise function remains unclear, several hypotheses suggest it may act as a reservoir for tubulin subunits during cytokinesis [20] or contribute to the detachment process [21]. A protein known as median body protein (MBP) was initially identified in the median body, but subsequent studies revealed its presence

in the ventral disk as well, where it is essential for maintaining the dome shape of the disk, thereby helping in attachment [8,17].

Cell division is a complex process in which trophozoites undergo binary fission along a longitudinal axis [22] (**Figure 4**). *Giardia* trophozoite cell division involves duplicating both diploid nuclei and multiple cytoskeletal structures. During the cell cycle, the ploidy of the *Giardia* trophozoites alternates between tetraploid (2 × 2 N) in G1 and octoploid (2 × 4 N) in G2 stage [23]. Upon encystation, a G2 cell (2 × 4 N) forms a cyst wall and divides its nuclei, producing a cell with four 2 N nuclei (4 × 2 N). DNA replicates again without cytokinesis, creating a 16 N cyst containing four 4 N nuclei [23]. Excystation releases a new form called excyzoite that divides into two G2 trophozoites (2 × 4 N), which re-enter the cycle and quickly undergo cytokinesis, yielding four G1 cells (2 × 2 N) [24].

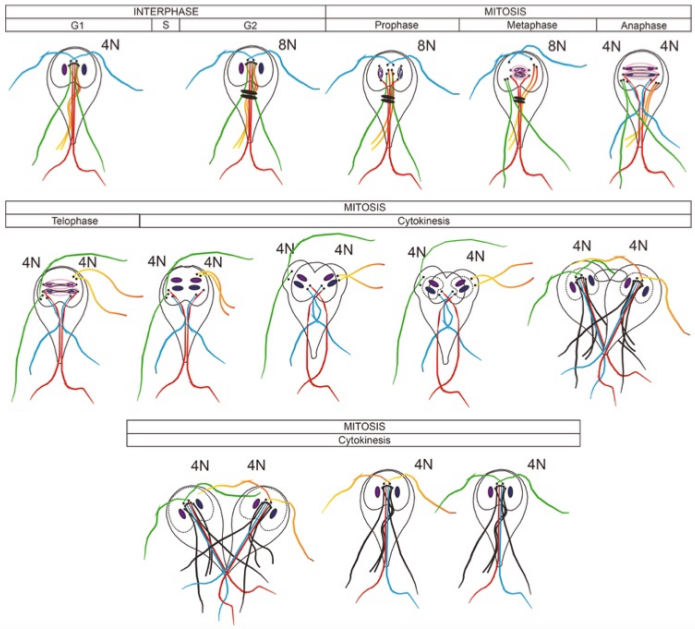

Figure 4. Dynamics of cytoskeletal structures during the *Giardia* cell cycle. This figure illustrates these changes from a dorsal view, highlighting structural transitions in the flagella. Parental flagella transform distinctly: anterior flagella (blue) become the right caudal flagellum in each daughter cell, posterior-lateral flagella (green) shift to anterior flagella in one daughter cell, and ventral flagella (yellow) transform to anterior flagella in the other. Meanwhile, caudal flagella (red) retain their form, consistently appearing on the left side in both daughter cells [23].

Throughout interphase, *Giardia* chromatin is decondensed, and basal bodies are positioned above the nuclei. Chromatin condenses in prophase, and although *Giardia* lacks H1 linker histone [25] and centrosome-associated proteins found in other organisms, it probably utilizes a simplified γ tubulin small complex (γTuSCs) for centrosomal microtubule nucleation [26]. During prophase, basal bodies duplicate, four spindle poles nucleate, and the nuclei realign along the dorsal-ventral axis. Spindles form without centromere alignment on a metaphase plate. During anaphase, spindle microtubules elongate along the cell's left-right axis, attaching to centromeres to segregate chromosomes [22,23]. Mitosis in *Giardia* is semi-open, with the nuclear envelope remaining intact as extranuclear spindles penetrate the nuclei [22]. Chromosome separation in *Giardia* occurs asymmetrically, with one nucleus progressing ahead of the other. Cytokinesis starts at the cell's anterior, forming a characteristic heart shape cell with four nuclei as flagellar motions drive the furrow [23]. This furrow progressively extends perpendicular to the chromosomal segregation axis, moving toward the posterior end, where the plasma membrane stretches. Unlike many other organisms, *Giardia* lacks a contractile ring due to the absence of myosin; instead, cytokinesis is facilitated by the flexions of the caudal flagella and the power strokes of the anterior flagella [20,22]. While actin plays a role in positioning microtubules and contributes to membrane and vesicle trafficking during cytokinesis [27], only minimal amounts of actin are present at the leading edge of the cleavage furrow. In contrast, RAB11—a small GTPase and actin-associated protein—plays a crucial role in defining the cleavage furrow, being concentrated at the ends of the developing axonemes. It may participate in the membrane remodeling necessary for the final separation of the two daughter cells [20]. Flagella in *Giardia* are inherited semi-conservatively, with anterior, ventral, and posterior-lateral flagella transforming or regenerating in daughter cells, establishing cell polarity. During mitosis, *Giardia*'s adhesive discs are newly formed and play a role in cytokinesis as they rotate, disassemble, and regenerate. This process completes when tail-to-tail connected daughter cells use their adhesive discs to separate fully [23].

The first *Giardia* genome was published in 2007 [28], and it has since been refined through optical mapping and, more recently, by extended read sequencing combined with optical mapping, providing a nearly complete

genome of the WB isolate (genotype AII) [29,30]. *Giardia* has five chromosomes (ranging from approximately 1 to 5 Mb) with a ploidy of four [8,24,31]. The entire genome is approximately 12 Mb and is compact, with minimal noncoding regions. Introns are rare, with only eight *cis*-spliced and five *trans*-spliced introns identified [30,32]. The genome contains 4,863 protein-coding genes, 2,099 of which are hypothetical proteins, while the remaining are annotated. Additionally, 306 pseudogenes have been identified [8,30]. Noncoding small RNAs (sRNAs) such as microRNAs (miRNAs), which regulate VSP gene expression, as well as endogenous siRNAs (endo-siRNA) and tRNA-derived sRNAs, have also been identified [33,34].

Giardia trophozoites are tetraploid with two diploid nuclei, and it was previously assumed that they reproduced asexually. Under this assumption, one would expect increasing allelic heterozygosity over time [8]. However, contrary to this expectation, the WB genome (genotype AI) exhibits an extremely low level of allelic heterozygosity (<0.01%), later revised to 0.03% [28,30]. In contrast, the GS isolate (genotype B) has a heterozygosity level of approximately 0.425% [35], while the DH isolate (genotype AII) shows an intermediate level (0.037%). The heterozygosity in dog genotypes (C and D) ranges from 0.52% to 0.58% [36]. The reasons for these differences remain unclear. An important question is whether heterozygosity is observed at the single-cell level. This question was investigated for genotype B using clones selected by limiting dilution and by sequencing single cells obtained through micromanipulation [37,38]. These studies confirmed the presence of heterozygosity at the single-cell level. Other studies of genotype B heterozygosity have shown that the frequency of silent substitutions in housekeeping genes complicates the classification of these organisms into specific subgenotypes or subassemblages [39,40]. In sexually reproducing organisms, allelic heterozygosity is typically limited by the need for chromosome pairing during meiosis. The levels of heterozygosity observed in *Giardia* (ranging from <0.01% to 0.5%) fall within the expected range for sexually reproducing organisms, raising the possibility that *Giardia* undergoes sexual reproduction. Support for this hypothesis could come from examples of recombination among isolates or evidence of meiosis at the cellular level [8]. In terms of proteomics and transcriptomics, relatively few 3D protein structures have been

experimentally determined. Sequence-based searches (e.g., BLAST or hidden Markov models) tend to have poor accuracy when amino acid identity is low. However, structure-based searches using machine learning, such as the I-TASSER software suite, have been applied to examine the set of approximately 5,000 *Giardia* proteins [41]. This approach resulted in the generation of 1,095 structural models with high confidence, including 212 hypothetical proteins [8]. Regarding nuclear and chromosomal structure, *Giardia* exhibits several distinctive chromosomal and cellular features compared to typical eukaryotes (**Table 2**). Studies using pulsed-field gel electrophoresis identified five chromosomes, as confirmed by cytogenetic methods, which show size variants due to differences in subtelomeric repeat regions [8,31]. *Giardia* trophozoites spend most of their time in the G2 phase of the cell cycle during *in vitro* cultivation [24]. In the G1 phase, the trophozoites have a ploidy of four, with two diploid nuclei that are generally identical in most respects, though a small degree of aneuploidy has been observed [42]. An interesting case involves the WB isolate and a cloned line, WBC6 [43], which was found to be aneuploid for chromosome 5, with three copies in one nucleus and one in the other [44]. The chromosome 5 in the haploid nucleus had a deletion in the subtelomeric region, and five genes from that region were expressed only from one nucleus. *Giardia* chromosomes are highly compacted compared to other eukaryotic chromosomes, containing 10-nm fibrils, 30-nm fibrils, and chromomeres [8].

Conventional eukaryotic organelle	**Equivalent *Giardia* organelle**
Nucleus	Two nuclei
Nucleolus	rDNA not organized in nucleolar pattern, but genes for nucleolus-localized proteins in genome
Mitochondrion	Mitosome
Golgi	Numerous features of Golgi transport
Endosome/lysosome	Peripheral vacuoles or vesicles
Peroxisome	Peroxisome-like proteins in cytoplasmic vescicles
Exosome	Exosome-like vesicles

Table 2. Comparison of conventional eukaryotic organelles and equivalent *Giardia* organelles [8].

For many years, it was thought that *Giardia* lacked a nucleolus, as fibrillarin, a protein involved in ribosome assembly, was found diffusely in the nuclei rather than in discrete nucleolar structures [45]. However, light and electron microscopy have now revealed structures consistent with a nucleolus, although there is no evidence of a conventional nucleolar organizing region [46–48].

In terms of DNA repair, *Giardia* has a DNA repair system in which the recombinase DMC1B functions similarly to Rad51 [49], and Rad52 also plays a role in DNA repair [50]. The significant differences in allelic sequence heterozygosity among genotypes, particularly between genotypes AII and B, suggest that *Giardia* may differ in the efficiency of its DNA repair systems [8]. Regarding transcription and translation, *Giardia* possesses genes for the eukaryotic RNA polymerases I, II, and III [8]. Although RNA splicing is relatively rare in *Giardia*, a spliceosome, along with core spliceosomal proteins and small nuclear RNA candidates, has been identified [51]. An early study suggested the absence of a 5-methyl-guanosine cap on mature mRNA [52], but subsequent studies confirmed that conventional capping occurs [53,54]. Given the very short 5' untranslated regions (UTRs), the first AUG codon is typically utilized for translation [8]. *Giardia* trophozoites contain a rudimentary organelle known as a mitosome, or mitochondrion-like organelle (MLO) [55–57]. The mitosome is a double-membraned organelle that contains iron-sulfur (Fe-S) clusters essential for the assembly of Fe-S-containing proteins but does not produce ATP [8]. The mitosome houses proteins involved in its own biogenesis, but no other known mitochondrial functions are present [58–60]. Unlike conventional mitochondria, the replication of *Giardia* mitosomes is linked to mitosis [61]. Recent evidence suggests that *Giardia* may have a rudimentary form of peroxisomes [8]. Proteins associated with peroxisomes, including acyl-CoA synthetase long-chain family member 4 (ACSL-4) and peroxin-4 (PEX-4), have been identified in the *Giardia* proteome [62]. *Giardia* has a minimal biosynthetic and energy production pathway, consistent with its status as an obligate parasite [63]. It relies on anaerobic energy metabolism, lacking cytochrome-mediated oxidative phosphorylation. Glucose is the primary energy source and is metabolized into acetate, ethanol, alanine, and CO2, with alanine being the major product under strict anaerobic conditions [8]. *Giardia* has limited endogenous amino acid synthesis and depends largely on amino

acids obtained from the host. The most studied pathway is the arginine deiminase pathway, where arginine is metabolized to produce ornithine, generating ATP from ADP [64,65]. Moreover, aspartate can serve as an energy source by being converted to pyruvate via a malate intermediate. In addition to purine recycling, *Giardia* also deepens on pyrimidine recycling, as do trichomonads [64]. Fatty acids are important for *Giardia*'s life cycle and metabolism, particularly during encystation. Cholesterol and bovine bile, which induce encystation, regulate the upregulation of genes involved in cholesterol biosynthesis [66]. Moreover, phosphatidylglycerol and phosphatidylethanolamine synthesis occurs in vegetative and encysting trophozoites [67].

Giardia trophozoites have long been thought to lack a Golgi apparatus, leading to the assumption that it may have evolved before this organelle developed in eukaryotes [68,69]. However, structures resembling the Golgi apparatus have been identified in encysting trophozoites [70], and protein transport inhibition studies using brefeldin A suggest that some Golgi-like functions are present [71]. It is now believed that the endoplasmic reticulum (ER) may fulfill many of the functions typically associated with the Golgi in *Giardia* [8,72]. Endocytosis in *Giardia* occurs via peripheral vesicles (PVs), which acidify to import essential materials and function as endosome-lysosomes [73,74]. These PVs interact with *Giardia*'s clathrin heavy chain and associated proteins, suggesting a role in membrane fusion during material import [75]. Exocytosis involves the secretion of variant-specific surface proteins (VSPs), which form a protein coat around each trophozoite [76–78]. Although the exocytosis system is not fully characterized, recent studies identified exosome-like vesicles in in vitro cultures, highlighting the role of ESCRT-associated proteins and ceramide in the formation of intraluminal vesicles inside PVs [79].

Regarding membrane and surface proteins, VSPs (formerly known as cysteine-rich proteins [80], trophozoite surface antigens [81], or trophozoite surface proteins [82]) undergo antigenic variation at rates as high as 10^{-3} per cell division [83]. These proteins are cysteine-rich (approximately 12% cysteine) [80] and heterogenous in size (from 2 to 6 Kb, corresponding to about 60 to 180 kDa for the translated protein) [8]. The VSP gene repertoire in the WB isolate is estimated to consist of 270–300 genes, making up 4% of the genome. These genes include 30 with tandem repeats and 14

arranged in head-to-head or tail-to-tail pairs [8]. The VSPs play a role in immune evasion and may also protect the trophozoites from intestinal proteases and aid in adaptation to different hosts [84–86]. The exact biological role of VSPs remains unclear, but their significant genomic representation suggests they are crucial [8].

Furthermore, *G. duodenalis* can be infected by *Giardia lamblia* virus (GLV), first identified about 35 years ago in a human-derived isolate known as HP-1 (Human Portland-1) [87–89]. GLV is a small, non-enveloped virus with a single double-stranded RNA (dsRNA) genome and is the only recognized member of the *Giardiavirus* genus within the *Totiviridae* family [90]. Other genera in the *Totiviridae* family include *Leishmaniavirus* and *Trichomonasvirus*, which infect protozoa, and *Totivirus* and *Victorivirus*, which infect fungi [87]. Recently, unclassified *Totiviridae* that infect arthropods, fish, and insects have been proposed to belong to the same clade as *Giardiavirus* [91–93]. The GLV-HP-1 genome (≈6.3 Kb), sequenced in the 1990s (GenBank ID L13218.1) [88], includes two overlapping open reading frames (ORFs) that encode for the capsid protein (CP): ORF1 for the CP and ORF2 for RNA-dependent RNA polymerase (RdRp) [88,90]. Unlike conventional mRNAs, GLV mRNA lacks the typical 7-methyl-G(5')ppp(5')G cap structure, relying instead on an internal ribosome entry site (IRES) to initiate translation of CP via a cap-independent mechanism [94]. The RdRp is produced as a fusion protein through a ribosomal frameshift during translation [95]. The GLV capsid, about 48.5 nm in diameter, has an icosahedral structure composed of 120 CP subunits, and the virus is unusually thermostable, allowing it to be transmitted extracellularly, a unique trait among *Totiviridae* protozoan viruses [90,96]. Approximately 30% of *G. duodenalis* isolates test positive for GLV [97–100], although only three full-length viral genomes have been deposited in GenBank: two from human and one from a dog isolate [101,102]. A recent study by Marucci *et al.* [90] uses high-throughput sequencing (HTS) to enhance understanding of *Giardia lamblia* virus (GLV) genomes, particularly focusing on different strains (HP, CAT, P2-MER, J17-10), which originate from humans [103], cats [104], pigs and sheep [99], respectively. Using Illumina sequencing, researchers uncovered significant insertions and deletions in GLV-HP compared to previously sequenced strains, suggesting that past sequencing errors may have skewed interpretations of GLV regulatory mechanisms [90]. *In vitro*

phenotypic differences were noted: GLV-CAT infections cause less severe cytopathic effects due to efficient viral release, while GLV-HP accumulation within host cells could increase pathogenicity. Additionally, the study identified a new potential RNA virus, GdRV-2, related to *Totiviridae*, which lacks an independent capsid protein and may belong to a novel virus family [90].

1.1.3 Life cycle

Giardia has a simple and direct life cycle (**Figure 5**): infection begins when a host ingests the cysts, which then pass through the stomach and reach the small intestine, particularly the duodenum. Here, the cysts undergo excystation, a process triggered by exposure to bile and a shift to a more alkaline pH [8]. *In vitro* experiments replicating this pH change and exposure to bile have shown high rates of successful excystation [10,105]. However, excystation can also occur at a neutral pH [16], making individuals with a higher gastric pH, whether naturally or due to medical treatments, more vulnerable to *G. duodenalis* infections [106]; two cases have been reported in which stopping proton pump inhibitors, which reduce stomach acidity, resolved gastric giardiasis without the need for antimicrobial therapy [107]. Within the host, trophozoites replicate in the small intestine, eventually differentiating into cysts subsequently excreted into the environment via feces. Animal studies suggest that these trophozoites tend to cluster in specific areas throughout the small intestine and even in the cecum. Encystation starts shortly after infection and reaches its peak within a week, occurring predominantly in these densely populated clusters [8,108]. The ability to induce encystation *in vitro* was first achieved using methods that involved the use of primary bile salts [109]. These methods were later adapted into a two-step process, which includes exposure to an alkaline pH of 7.8 and porcine bile [110]. Other techniques for inducing encystation involve high-bile mediums [111] or cholesterol deprivation [112]. Early in the encystation process, specific vesicles known as encystation-specific vesicles (ESVs), develop [70]. ESVs share properties with the Golgi apparatus and are involved in protein transport, particularly of cell wall proteins 1 to 3 (CWP1 to CWP3). During encystation, trophozoites become rounded, and some of their key cytoskeletal components disassemble [8,113].

Two cycles of chromosome replication and a single nuclear division result in mature cysts, each containing four nuclei, all in a 4 N state [8].

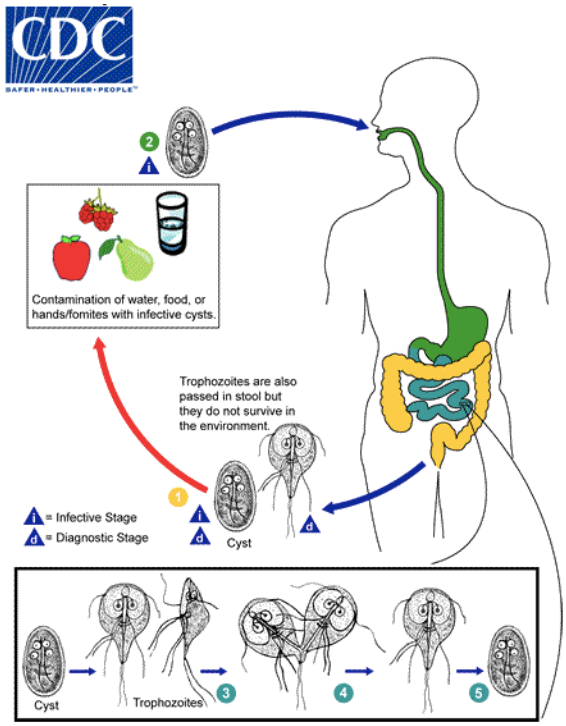

Figure 5. Life cycle of *Giardia duodenalis*.

When favorable conditions are encountered, an opening at one end of the cyst allows the release of the flagella and the cell body of the excyzoite, which divides twice to produce four trophozoites, each with two diploid nuclei (4 N) [24]. The genome of *Giardia* contains 27 cysteine proteases from the CA clan [114], with 25 of these being expressed. The most abundant is GlCP2 (CP2), a cathepsin B-like enzyme involved in both excystation and encystation. CP2 is present in ESVs and can cleave recombinant CWP2 into a 26-kDa fragment, like what is found in encysting cells [114–116]. The roles of other cysteine proteases remain unclear. Research on the transcriptomics of encystation using techniques like serial analysis of gene expression (SAGE) and microarrays has shown that genes coding for CWP1 to CWP3, a high cysteine nonvariant cyst protein (HCNCp), and the transcription factor Myb are significantly upregulated within the first three hours [117,118]. HCNCp was the first identified protein from a family of high-

cysteine membrane proteins (HCMPs or MCMPs). Other genes, including those with Myb-binding sequences, are upregulated after seven hours [8,119]. Proteomic studies using tandem mass spectrometry have indicated that the variety of variant-specific proteins (VSPs) decreases four hours after encystation begins, compared to baseline levels, along with multiple changes in metabolic and cytoskeletal proteins [8,120]. The exact mechanisms that initiate encystation are not fully understood. However, some factors have been identified that can inhibit the process, such as nitric oxide, histone deacetylase inhibitors, and lactoferrin [121–123]. Additionally, a study on a specific Rho family GTPase found in *Giardia* (Rac) demonstrated that this enzyme is localized to the ER and ESVs, and its expression promotes CWP1 production. This CWP1 then accumulates in the extracellular environment and is used by nearby trophozoites to support encystation. This mechanism may explain why encystation is often observed in clusters of *Giardia* organisms [8,124]. All the life cycle of *Giardia* can be experimentally completed *in vitro* [125] providing new opportunities to study its different forms and associated antigens.

1.2 Epidemiology and risk factors

Giardiasis was regarded a relatively minor disease until 1971 [126]. Between 1906 and 1946, research on *Giardia* was limited, with only a few publications emerging each year. However, following World War II, interest in the field increased, with annual publications rising to 20-40 between 1947 and 1961 [127]. The identification of the antigiardial effects of metronidazole in 1961 [128], further accelerated research, leading to approximately 80 publications annually between 1967 and 1970 [127]. Research in the 1950s and 1960s primarily focused on experimental human infections to establish the infectious dose and the axenic culture of *G. duodenalis* [129,130].

Currently, *G. duodenalis* is globally distributed and represents a significant public health concern for both humans and animals. Its distribution and prevalence are highly variable, depending on environmental conditions and socio-economic factors. The cysts of *G. duodenalis* exhibit remarkable resilience, capable of surviving for extended periods, sometimes months, in humid environments. Consequently, regions with high humidity or tropical climates tend to report higher prevalence rates of giardiasis, as the

cysts remain viable in the environment for longer durations. Conversely, prevalence rates are generally lower in temperate climates, where environmental conditions are less conducive to cyst survival.

In low-income countries, where access to clean water and sanitation is often limited, infection rates can reach alarming levels. Studies indicate that in high-income countries, the prevalence of *G. duodenalis* infections in humans ranges from 2% to 7%, while in low-income countries, this range can extend from 20% to 60%. This disparity reflects the significant differences in living conditions, hygiene practices, and access to safe drinking water [131,132].

Waterborne transmission is the most widely recognized route of giardiasis spread, with several outbreaks attributed to drinking water contaminated by *Giardia* cysts. A global review of waterborne protozoan parasitic outbreaks between 2011 and 2016 reported that *Giardia* spp. was responsible for 37% (142) of the 381 documented outbreaks [133,134]. These 142 outbreaks accounted for 1,110 cases of giardiasis, with the majority of cases reported in the United States (U.S.) and New Zealand, and a single case from South Korea and one from Belgium [127]. The outbreaks were predominantly linked to untreated or contaminated drinking water, as well as recreational water sources. The incidence of waterborne outbreaks increased substantially, from 199 outbreaks between 2004 and 2010 to 381 between 2011 and 2016, likely reflecting improvements in data collection and surveillance infrastructure (Efstratiou, Ongerth and Karanis, 2017). However, it is important to note that waterborne infections remain under reported in many developing countries, where the risk of infection is greatest, resulting in an underestimation of the true burden of the disease (Efstratiou, Ongerth and Karanis, 2017). Investigations into several waterborne outbreaks in the United States have implicated beavers as potential sources of contamination [135–137]. Coupled with frequent giardiasis cases reported among backpackers and campers consuming untreated stream water, this association has led to giardiasis being colloquially referred to as "beaver fever" [127,138,139].

Although less common, foodborne transmission of giardiasis has become more recognized in recent years; *G. duodenalis* ranks 11th in a global listing of foodborne parasites by importance [140]. Foodborne transmission is increasingly linked to the globalization of the food trade, international

travel, and changing consumer habits [141]. In the U.S., it is estimated that 7–15% of domestically acquired giardiasis cases are foodborne [142,143]. *Giardia* cysts have been found on various food items, including fresh produce, dairy products, meat, shellfish, and processed foods [134,144].

Many surveillance studies worldwide have reported *Giardia* contamination on fresh produce such as leafy greens, herbs, berries, green onions, carrots, and tomatoes [141,144,145]. Although relatively few foodborne outbreaks of giardiasis have been reported globally, the majority have occurred in the U.S., where 38 outbreaks were documented between 1971 and 2011 [146]. The true number of foodborne outbreaks is likely underestimated due to inadequate surveillance and reporting in many countries [144]. While the specific foods involved in these outbreaks are often not clearly identified, a wide range of items have been implicated, with fresh produce—such as salads, raw vegetables, and fruit—being most frequently associated. Other implicated foods include canned salmon, raw oysters, ice, and various processed foods, including noodle salad, chicken salad, dairy products, sandwiches, and tripe soup [134,141,144,146,147]. Fresh produce can become contaminated with infectious *Giardia* cysts at multiple stages, from farm production to consumer handling. At the farm level, contamination may occur during production, harvesting, packaging, or transportation. Poor personal hygiene among farm workers, such as contact with contaminated hands or equipment, can lead to direct contamination. Additionally, the use of animal manure as fertilizer can introduce *Giardia* cysts, particularly when livestock or other animals have direct access to the cultivated areas, thereby increasing the risk. Indirect contamination can also occur through the use of faecally contaminated water for irrigation, pesticide mixing, or produce washing [134,145]. The direct contamination of fresh produce by infected food handlers or individuals who have been in contact with infected persons represents another significant risk factor, as demonstrated by several outbreak investigations [141,144,146,147].

Giardiasis, in addition to being transmitted indirectly through waterborne or foodborne pathways, can also be spread via direct contact. Direct transmission occurs through person-to-person, animal-to-animal, or zoonotic routes. Among these, person-to-person transmission represents a significant source of infection, particularly in environments such as daycare centers, nursing homes, and other institutional settings where hygiene

practices may be insufficient [146]. Moreover, sexual transmission of giardiasis has also been reported [134,148,149].

It is now well-established that both humans and animals can be infected with a diverse range of *G. duodenalis* genotypes. Assemblages A and B are primarily responsible for giardiasis in humans, with assemblage B being more commonly associated with human infections. However, in regions such as South America and the Middle East, human infections with assemblage A are more prevalent than those caused by assemblage B. Although assemblage E is generally considered specific to hoofed livestock, it has recently been reported in over 50 human giardiasis cases in countries such as Brazil, Egypt, Vietnam, Australia, and New Zealand [150–154]. Assemblages C, D and F are far less commonly detected and have mostly been identified through genotyping at a single genetic locus [155,156].

Most mammals are infected with assemblages of *G. duodenalis* that are specific to animals. This is particularly true for farm animals and companion animals [157,158]. Among farm animals, ruminants such as cattle, sheep, and goats are primarily infected with the livestock-specific assemblage E, though assemblages A and B are occasionally detected. Wild ruminants like deer can be infected with assemblage E, but are more frequently infected with assemblage A [159–161]. Pigs are more commonly infected with assemblages A, B, and E [162–164]. Among companion animals, dogs are primarily infected with the canine-specific assemblages C and D, with some occurrences of assemblages A and B, while cats are mostly infected with the feline-specific assemblage F, although assemblages A and C are also found [157,158]. Rodents such as rats and mice are typically infected with assemblage G [165–168]. Several animal groups are commonly infected with assemblage B, which poses significant zoonotic concerns. This includes equines, rabbits, and chinchillas, many of which are increasingly kept as companion animals and pets. Assemblage B is the dominant genotype in rabbits and chinchillas [169–173], while both assemblages A and B are frequently found in horses and donkeys [174–177]. Other animals with a high prevalence of assemblage B include farmed masked palm civets and bamboo rats [178,179], as well as captive raccoons [180], foxes [181], and sea lions [182]. Farmed raccoon dogs, however, are predominantly infected with assemblages C and D [180,183]. Assemblage B is especially common in non-human primates [127]. Given that many different mammal species, including

those harboring Assemblages A and B, can serve as hosts for *G. duodenalis*, the possibility of zoonotic transmission has garnered considerable attention. However, direct evidence supporting zoonotic transmission remains limited, and the role of animals in human infections is not as well understood [134]. It is important to recognize that the mere presence of common genotypes in both humans and companion animals does not necessarily indicate zoonotic transmission [157]. Nevertheless, the high prevalence of *G. duodenalis* in livestock, combined with the presence of zoonotic assemblages, raises concerns about potential transmission to humans, either through direct contact (e.g., among farmers, veterinarians, and animal handlers) or indirectly through contamination of food and water [184,185]. Reverse zoonotic transmission (zooanthroponosis) has also been suggested, particularly between humans and wildlife or companion animals [186–188].

In pets, *G. duodenalis* is primarily transmitted to dogs and cats through direct contact with contaminated environments, particularly in multi-animal households or settings such as animal shelters and boarding facilities where animals are in close proximity. Transmission can also occur through the ingestion of cysts from contaminated water, food, or feces, paralleling the transmission dynamics observed in humans. Additionally, asymptomatic carriers in animals may serve as reservoirs, further complicating efforts to control the spread of the parasite. The prevalence of *G. duodenalis* in dogs and cats exhibits geographical variation, with higher infection rates typically observed in areas with inadequate sanitation and poor hygiene. In the United States, the prevalence of *G. duodenalis* in pets ranges from 5% to 15%, whereas in Europe, the prevalence is higher, ranging from 14% to 30% in dogs and 11% to 26% in cats [189–191].

1.3 Pathogenesis and clinical manifestations

1.3.1 Pathogenesis

Giardia duodenalis is a non-invasive pathogen of the small intestine, colonizing both the lumen and the epithelial surface [192]. In humans, although trophozoites are typically found in the proximal small intestine, they have also been observed in the stomach, distal small intestine, cecum, and pancreas [193]. In animal models, the trophozoites predominantly colonize the proximal small intestine but can also be detected in the ileum o cecum [108]. *Giardia* can cause a wide range of clinical signs, ranging from asymptomatic to severe, including chronic diarrhea, weight loss, abdominal pain, and post-infectious complications (e.g., irritable bowel syndrome (IBS), chronic fatigue, and growth stunting) [8,134]. The diversity in clinical outcomes likely results from variations of host factors, parasite characteristics, or microbiota composition, complicating efforts to fully understand the mechanisms underlying these manifestations [8]. However, significant progress has been made over the past two decades in uncovering some of these mechanisms, with recent reviews providing comprehensive insights [192,194–196].

The trophozoites of *G. duodenalis* adhere to the small intestinal mucosa, leaving a visible imprint upon detachment from the epithelial surface [197], prompting speculation about mechanical attachment as a potential mechanism of pathogenesis although no evidence currently supports this hypothesis [8]. Instead, existing data suggest that a combination of secrete proteases, other *Giardia* factors, host immune responses, and interactions with the intestinal microbiota contribute to the diverse clinical presentations [194,198]. Notably, in symptomatic giardiasis patients undergoing biopsies, villous flattening is often observed without clear inflammatory changes although in some cases inflammatory changes appear distinct from the sites of trophozoite attachment [199].

In the pathogenesis of *Giardia*, excretory-secretory products (ESPs), particularly cysteine proteases (CP), play a crucial role [198]. Although serine proteases are also present in ESPs, cysteine proteases dominate the proteolytic activity [200]. These enzymes are primarily involved in regulating encystation and excystation but also significantly affect interactions between trophozoites and the host's intestinal epithelium [8].

Trophozoites disrupt intestinal tight junctions through several mechanisms, including the action of cysteine proteases [202,206]. For example, a study investigating the effect of CP-2 on an intestinal epithelial cell line (IEC-6) demonstrated that the protease induced cell junction damage, which was mitigated by the protease inhibitor E-64 [203]. Additionally, CP-2 has been shown to cause apoptosis and damage villi [204]. Moreover, the intestinal mucus layer, a critical component of innate immunity, is notably affected by cysteine proteases. These enzymes degrade *in vitro* MUC-2 (a key human mucus protein) while also increasing MUC-2 gene expression in goblet-like cells, suggesting a potential depletion of the intestinal mucus reserve [205]. This finding aligns with observations in infected wild-type mice, where mucus thinning occurred, and MUC-2-deficient mice experienced weight loss upon infection. Furthermore, bacterial translocation increased in infected wild-type mice [205]. While cysteine proteases are a major focus, other ESPs likely contribute to *Giardia*'s pathogenesis. A systematic analysis of *Giardia*'s secretome, using two isolates (WB from subassemblage AI and GS from Assemblage B) in co-culture with intestinal epithelial cells (IECs) derived from Caco-2 cells, identified dominant ESPs involved in metabolism. These included proteins related to glycolysis, arginine metabolism, phospholipid remodeling, and nucleotide salvage, as well as proteins linked to encystation, variant-specific surface proteins (VSPs), high-cysteine membrane proteins (HCMPs), alpha-giardins (annexins), and tenascins (extracellular matrix glycoproteins) [8]. While the general classes of proteins were similar across isolates, the number of identified proteins varied [206]. The interaction between *Giardia* and IECs also triggered notable host responses, including chemokine production for immune cell recruitment, alterations in glucose and lipid metabolism, and induction of apoptosis. A transcriptomic analysis of WB trophozoites interacting with IECs revealed that HCMPs exhibited the most substantial upregulation, though their expression patterns varied [207]. Similarly, a proteomic study focusing on tenascins identified comparable results across WB and GS isolates, underscoring their potential role as virulence factors. However, the function of *Giardia* tenascins remains poorly characterized [208]. Arginine metabolism plays a critical role in *Giardia* pathogenesis. The parasite's arginine deiminase, a cytoplasmic enzyme secreted during interactions with IECs, depletes arginine [209],

reducing nitric oxide production [210,211], inhibiting IEC proliferation [212], and impairing T-cell proliferation [210]. This depletion also modulates the immune response by increasing TNF-α and reducing IL-10 levels in dendritic cells [213]. Arginine deiminase is thus considered both a virulence factor and a contributor to trophozoite energy metabolism [8].

Even the immune system plays an important role in the pathogenesis of *Giardia*. Early assumptions about the central role of antibody responses in immunity against *Giardia* infections were supported by reports of prolonged giardiasis in individuals with total immunoglobulin or IgA deficiency. However, these observations were not well-documented, and the understanding of this immune response has since become has grown increasingly refined [8]. Evidence for the importance of IgA includes its robust response in infected humans, particularly targeting semi-conserved regions of variant-specific surface proteins (VSPs) [214]. The role of antigenic variation in evading host immunity remains a subject of interest. While early studies in gerbils showed limited VSP changes after an initial shift within seven days of infection [86], data from human infections suggest that antigenic variation plays a more substantial role in persistence within the host [84]. Subsequent research strongly supports this hypothesis. A vaccination study using a WB isolate modified to express multiple VSPs simultaneously (via RNAi suppression) demonstrated effective immunity against reinfection in gerbils [215]. Similarly, immunization with a vaccine containing multiple VSPs protected both gerbils and domestic animals [216]. This suggests that VSPs play a dual role in eliciting immune responses and facilitating survival through antigenic variation [8]. The Th17 immune response, particularly IL-17A, has emerged as a critical component of immunity to *Giardia*. IL-17A has been observed in mice infected with both *G. duodenalis* [217] and *G. muris* [218], where it enhances the IgA response [219]. IL-17 receptor A (IL-17RA) regulates local IgA production during infection, as evidenced by impaired immune responses in IL-17RA knockout mice [220]. Studies in human travelers and Brazilian children have shown correlations between Th1/Th17 responses and infection outcomes, suggesting parallels between mouse models and human immunity [221,222]. Impaired Th17 responses, as seen in BALB/c mice, result in poor clearance of *Giardia*, while overly robust responses may cause harmful inflammation, highlighting the need for balanced immunity [223]. This may

explain the variability in infection symptoms and clearance across hosts. Mast cells also contribute to the immune response by controlling infection [195], though they may play a role in the abdominal cramping associated with symptomatic giardiasis [224]. Their activation might be mediated by *Giardia*'s arginine deiminase or its byproduct, citrulline [225]. Macrophages, which accumulate in the intestinal lamina propria during infection, produce arginase 1 and nitric oxide synthase 2 [226]. However, macrophage depletion does not impair trophozoite clearance, leaving their exact role unclear [227]. Interestingly, *Giardia* can attenuate the host inflammatory response. Extracts of *G. duodenalis* increase IL-10 production and decrease IL-12 in dendritic cells via the PI3K pathway [228,229]. In IL-10-deficient mice, *G. muris* infection led to colitis dependent on Myd88 signaling and facilitated by the intestinal microbiota [230]. Conversely, *Giardia* infection can mitigate intestinal inflammation caused by other pathogens, such as *Citrobacter rodentium*, through NLRP3 inflammasome activation and enhanced antimicrobial peptide production [78,231]. In humans, *G. duodenalis* has been shown to reduce neutrophil infiltration in Crohn's disease biopsies [232] and degrade IL-8 during co-infections with *Salmonella* [233]. These findings illustrate the complex interplay between *Giardia* and the host immune system, revealing both protective and regulatory mechanisms that influence infection outcomes [8] .

Over the past decade, the importance of the intestinal microbiota in health and disease has gained recognition. Recent advancements in next-generation sequencing have allowed for detailed assessments of the microbiota's role in various conditions, including malnutrition, inflammatory diseases, and metabolic disorders [234,235]. In the context of *G. duodenalis*, studies have highlighted the significant role of the microbiota. For example, germfree animals do not develop disease from *Giardia*, despite being susceptible to infection [236,237], and differences in microbial flora influence susceptibility to infection [238]. Research using a mouse model infected with Assemblage B (GS isolate) showed that antibiotic treatment increased the number of *Giardia* organisms in the small intestine, with shifts in the microbiota towards *Proteobacteria* and away from *Firmicutes*. In contrast, another study involving human microbiota exposed to *Giardia* trophozoites (genotype unspecified) and transferred to germfree mice revealed an enrichment in *Firmicutes*, particularly *Clostridiales*

species [8]. These findings highlight the complex interactions between *Giardia* and the microbiota during infection. Further research using the GS isolate showed that *Giardia* infection decreased sucrase activity, which was mitigated by broad-spectrum antibiotics that did not target *Giardia* directly [239]. Antibiotics also prevented pathological CD8 T-cell activation, indicating that the microbiota influences the immune response to giardiasis [239]. *Ex vivo* studies demonstrated that *Giardia*'s secretory proteases disrupted microbial biofilms, enhancing proinflammatory cytokine production in lymphocytes and altering epithelial cells [8]. These changes included reduced ZO-1 protein expression, increased CXCL-8 production, and heightened TLR4 expression, suggesting that giardiasis pathogenesis involves a TLR4-mediated mechanism, potentially linking it to IBS [240]. In another mouse model, disruption of tight junctions persisted after *Giardia* resolution, allowing bacterial influx and neutrophilic inflammation [241]. Conversely, TLR2-deficient mice displayed enhanced *Giardia* clearance with increased proinflammatory cytokine production and no changes in microbiota [242]. Human studies have further explored the connection between *Giardia* and the microbiota. A study in the Ivory Coast found a dysbiotic microbiota in individuals with giardiasis, characterized by a decreased *Faecalibacterium prausnitzii*:*Escherichia coli* ratio [243]. Studies in Colombia, Argentina, and the Global Enteric Multicenter Study cohort showed a distinct microbiota in children with *Giardia*, marked by an increased prevalence of *Prevotella* spp. and a decrease in *Gammaproteobacteria* [244–247]. These studies suggest a potential association between *Giardia* infection and microbiota changes, though further research is needed to establish causality [8]. Additionally, studies in animal models of post-giardiasis IBS showed persistent visceral hypersensitivity and intestinal barrier disruption, with the expression of c-fos serving as a marker of IBS pain [248]. Investigations into the synergistic effects of malnutrition and *Giardia* demonstrated that a protein-deficient diet worsened *Giardia*-induced malnutrition and immune dysfunction, supporting the complex interactions between *Giardia* and host factors like nutrition [249]. Another study revealed that *Giardia* interfered with the clearance of enteroaggregative *E. coli*, highlighting the potential for persistent infections [250]. These findings from human and animal studies underscore the complex interactions between *Giardia*, the microbiota, and

the host, highlighting their implications for malnutrition and long-term gastrointestinal dysfunction.

1.3.2 Clinical manifestations

In humans, asymptomatic infections with *G. duodenalis* are common, but symptoms including diarrhea, bloating, greasy stools, flatulence, epigastric pain, nausea, and vomiting can occur after a 1–2 week incubation period [192,251]. The acute phase of giardiasis typically lasts 1–3 weeks, but symptoms may persist for months. Although most infections resolve spontaneously, recurrences are frequent. However, most infections are subclinical, particularly in highly endemic areas where children are infected early in life [134].

Studies in Peru showed frequent reinfections after treatment, without associated symptoms or growth impacts [252,253]. Other studies in endemic regions have noted inconsistent associations between *Giardia* and diarrhea or malnutrition, with some research even suggesting a protective effect against diarrhea [253–255]. Nonetheless, evidence links *G. duodenalis* infections to malnutrition and growth stunting in children, particularly during critical developmental windows [256–264]. The MAL-ED study observed increased malnutrition indicators, including stunting and impaired nutrient absorption, across sites, although *Giardia* did not elevate fecal inflammatory markers as other pathogens did [261–263].

Furthermore, giardiasis can lead to post-infectious complications such as irritable bowel syndrome (IBS), functional dyspepsia, and chronic fatigue syndrome (CFS) [8,134]; in a large outbreak in Norway, 46% of affected individuals developed IBS and CFS within three years, with symptoms persisting in many even after six years [265–267], and U.S.-based studies have also confirmed an increased risk of IBS following giardiasis [268,269], potentially exceeding the risks associated with other gastrointestinal pathogens [270]. Extra-intestinal complications, such as reduced cognitive function, ocular pathologies, arthritis, allergies, hypokalemic myopathy, urticaria [271], and even cancer, have also been reported [251,272]. These complications are not due to direct invasion by the parasite, and the mechanisms linking them to giardiasis remain unclear [193].

In animals, giardiosis is often asymptomatic, but domestic animals may show signs such as diarrhea, weight loss, and poor growth [134,187]. In

livestock, the disease can cause significant economic losses due to reduced productivity, poor growth, and even death [2]. Subclinical infections in livestock can also impair productivity [185]. In dogs and cats, infections are frequently asymptomatic, although diarrhea is common in puppies and kittens [273]. The variability in clinical outcomes among hosts may be influenced by differences in parasite virulence, host nutritional status, gut microbiota composition, coinfections with other pathogens, and host immune responses [134,157,187,192,251]. However, there is no consistent correlation between the *Giardia* Assemblage involved and clinical manifestations: for instance, studies in Spain, the UK, Turkey, Egypt, Bangladesh, Australia, and Peru associate symptomatic cases mostly with *G. duodenalis* Assemblage A (particularly AII); in contrast, research in the Netherlands, UK, Spain, Saudi Arabia, Egypt, Ethiopia, Malaysia, and Cuba links most symptomatic infections to Assemblage B [157,274]. Intra-assemblage variation likely contributes to these differing findings [134,157,274].

1.4 Diagnosis

In the diagnosis of giardiasis, a variety of methods are available, which can be categorized as follows: i) copromicroscopic examination; ii) direct immunofluorescence assay (DFA); iii) rapid immunochromatographic tests (ICTs) or enzyme-linked immunosorbent assays (ELISA); and iv) molecular techniques, such as polymerase chain reaction (i.e. PCR).

i) Giardiasis is diagnosed by identifying cysts or, less commonly, trophozoites using **microscopic examination** of a stool sample, such as those processed through flotation zinc sulfate solution (specific gravity 1.180) or formalin-ethyl acetate sedimentation. Direct microscopic examination, although being a relatively non-sensitive technique, is recommended when examining fresh feces, especially if they are liquid or soft, primarily to detect the motility of trophozoites. The slide is prepared by diluting a small amount of feces in one or two drops of saline solution and then examining the suspension under a 22 x 22 mm or 24 x 24 mm coverslip, or even larger sizes. It is also possible to prepare an additional suspension using a drop of iodine solution or Lugol's solution (1:5), which helps to better highlight the internal structures of cysts. Permanent staining techniques, including trichrome or iron hematoxylin staining, can also be applied after stool preservation in media like polyvinyl alcohol (PVA), 10% formalin, sodium acetate-acetic acid-formalin (SAF), or merthiolate-iodine-formalin (MIF) [272,275]. Since cyst shedding can be intermittent, multiple stool samples collected over time (e.g., three specimens collected every 2–3 days) are recommended to increase diagnostic accuracy [134,272,275]. It is important to notice that microscopy cannot differentiate between *Giardia* species and Assemblages, a capability provided by molecular techniques [134]. While copromicroscopy is cost-effective due to minimal equipment and reagent requirements, it is labor-intensive, subjective, and demands considerable expertise, since there may be artifacts that can be confused with *Giardia*'s cysts [8].

ii) The application of direct fluorescent antibody staining, also known as the **direct immunofluorescence assay** (DFA), has enhanced the

sensitivity and specificity of microscopic analysis. However, this method remains time-consuming and requires skilled personnel [276]. Direct immunofluorescence assay is one of the most sensitive and specific techniques for diagnosing *Giardia duodenalis*. It relies on monoclonal antibodies labeled with a fluorochrome (e.g., fluorescein) that specifically bind to antigens on the surface of *Giardia* cysts and/or trophozoites in stool samples. Initially validated in humans, DFA was subsequently adapted and validated for use in animals and is now considered the gold standard diagnostic test, with a sensitivity of 99.4% (95% CI: 98.86–99.7) and a specificity of 99.8% (95% CI: 99.3–99.9) in dog samples, and even higher values reported for cats [277].

iii) To address some limitations of microscopy, rapid fecal antigen detection tests, such as **immunochromatographic tests** (ICTs) and **enzyme-linked immunosorbent assays** (ELISA), have been developed and are widely utilized [134,275,278]. However, each method has specific limitations and optimal use cases. Immunochromatographic tests have gained popularity among veterinary practitioners for the initial diagnosis of protozoan infections, thanks to their straightforward procedure, low resource requirements, and the speed of obtaining results on-site [279]. They provide qualitative results within minutes and require minimal technical expertise. Nonetheless, ICTs may exhibit lower sensitivity when detecting low parasite burdens, as seen in asymptomatic carriers, and they cannot quantify antigen levels, which limits their utility for monitoring disease progression or treatment efficacy. Moreover, the antigens can be detected for a long time, making the test positive even though the animal no longer sheds *Giardia* cysts. This makes the test unsuitable for post-treatment monitoring. In the end, they may also show occasional cross-reactivity with other parasites, leading to false positives [277,279]. On the other hand, ELISAs offer higher sensitivity and the ability to quantify antigen levels, making them suitable for detecting low-intensity infections and for use in research or disease monitoring. They are particularly effective for batch processing in large-scale studies or surveillance programs. However, ELISAs require specialized laboratory

equipment, trained personnel, and more time, making them less practical for field use or emergency situations. Their cost can also be a limiting factor, and variability in *Giardia* antigen expression might affect their sensitivity in certain scenarios. While ICTs are preferable for quick, qualitative diagnosis, especially in symptomatic cases, ELISAs are better suited for comprehensive, high-sensitivity analysis in clinical or research settings [280].

iv) **PCR-based methods** are more objective than microscopy, enabling species and assemblage identification through DNA sequencing, detection of multiple targets via multiplexing, and quantification of results [274,276]. Consequently, molecular approaches to *Giardia* testing offer improved insights into transmission patterns and the zoonotic potential of isolates. For accurate diagnosis or characterization of *Giardia* using PCR, stool samples should either be stored unpreserved in a refrigerator or freezer or collected with a preservative compatible with molecular methods and kept at room temperature [134]. Commercially available fixatives and preservatives can be used for this purpose. Alternatively, stool samples may be mixed with 2.5% potassium dichromate and stored for up to one month before PCR testing [134,281]. Genes commonly targeted for identifying and characterizing *Giardia* at the molecular level include small subunit ribosomal DNA (SSU-rDNA), internal transcribed spacers (ITS1 and ITS2), β-giardin, triosephosphate isomerase (TPI), and glutamate dehydrogenase (GDH) [186,187,274]. Among these, the SSU-rDNA gene, being a highly conserved multi-copy gene, often results in higher amplification success but lacks the resolution needed for subtyping provided by single-copy genes [187]. However, different target genes may yield discordant results in assemblage identification, emphasizing the need for more refined tools [186]. Identifying a *Giardia* genotype often requires a multi-gene approach for greater accuracy and improved resolution when distinguishing between genotypes, as some genes are more variable than others. A single gene may not be sufficient to differentiate all genotypes, especially in cases of co-infection or when genetic characteristics are similar. Common markers used in multi-gene approaches include the glutamate dehydrogenase (gdh), β-giardin

(bg), and triosephosphate isomerase (tpi) loci. The sequence analysis of short fragments of the 18S rRNA locus should be avoided [186]. Ryan *et al.* [276] discussed several emerging technologies addressing limitations in current diagnostic methods for enteric parasites. For instance, commercial multiplex PCR panels now enable the simultaneous detection of multiple common enteric protozoan parasites, including *Giardia*, and many of these assays rely on real-time PCR for rapid, high-throughput detection and quantification of target DNA [276]. Next-generation sequencing (NGS), a high-throughput technology, facilitates whole-genome or targeted sequencing, allowing for the discovery of novel genotypes and detection of mixed infections. NGS is increasingly employed not only for diagnosis but also in epidemiological studies and outbreak investigations [134,158,187,276]. Draft genomes for various *G. duodenalis* isolates from Assemblages A, B, and E are now available [2,134,274,282,283]. Recently, reference-quality genomes of three *Giardia* isolates were constructed using a hybrid approach that combines NGS and third-generation long-read sequencing [134,284].

In addition to these primary diagnostic approaches, there are other, less commonly used methods, including endoscopic biopsy, the Entero-test (string test), and serological detection of antibodies. Endoscopic biopsy and examination of duodenal contents may be performed, accompanied by immediate microscopic analysis of the intestinal material to detect trophozoites [8]. An alternative diagnostic method is the Entero-Test, which involves the patient swallowing a gelatin capsule enclosing one end of a string, while the other end is taped to their cheek. As the capsule dissolves, the string continues through the digestive tract via peristalsis into the duodenum. After approximately 4 hours, the string is retrieved, and the mucus at its end is examined for parasites [8,285,286]. Regarding serum antibody test, it has been utilized in research to investigate the epidemiology of giardiasis, but it lacks sufficient sensitivity and specificity for clinical diagnosis [8].

1.5 Therapy, prevention and control

1.5.1 Therapy

Giardia duodenalis infection presents a wide spectrum of clinical manifestations, ranging from asymptomatic cases to prolonged and severe diarrhea; this variability makes it challenging to decide who should receive treatment for giardiasis: for example, in highly endemic areas, treating asymptomatic individuals is probably no value [8]. Despite the association between *Giardia* infection and growth stunting, high reinfection rates can limit the effectiveness of treatment in eradicating the infection [253]. Furthermore, the long-term impacts of nitroimidazoles and other antibiotics on antimicrobial resistance and the microbiota remain insufficiently understood, making treatment in asymptomatic cases harder to justify [8]. In low-prevalence settings, treating asymptomatic individuals could potentially reduce transmission, but there is no supporting data for this approach. Conversely, symptomatic giardiasis typically warrants treatment, as the condition often persists without intervention [8] and it can cause post-infectious complications. *Giardia* has a mechanism for antigenic variation which allows trophozoites to evade the immune response of the host, but creates challenges for the development of vaccines; actually, no effective and/or approved vaccine is available and pharmacotherapy is the only option to treat giardiasis [287]. In humans, effective approved drugs consist of a classes of six compounds (**Figure 6**), namely 5-nitroimidazoles and benzimidazoles derivatives, quinacrine, furazolidone, paromomycin, and nitazoxanide [287]. Metronidazole is the 5-nitroimidazole prototype and it became widely used in the 1960s for giardiasis and other infections caused by anaerobic protozoa or bacteria [288]. It is still considered the first-line antigiardial agent with recovery rates exceeding 90% [272,289]. Despite its efficacy, this molecule may exhibit adverse side effects (e.g., headache, nausea, glossitis, neutropenia) and has carcinogenic, teratogenic, and embryogenic properties [289,290]. Metronidazole is reduced to toxic intermediates with multiple effects, including DNA reactivity; pyruvate-ferredoxin oxidoreductase (PFOR) and thioredoxin reductase (TrxR) are among the proposed mechanisms of action, though additional pathways are likely involved [288,291–293]. Metronidazole is typically prescribed for 5 to 10 days, with a large number of studies confirming the high efficacy for

giardiasis treatment although the required treatment duration can hinder compliance and tolerability. Single-dose regimens have been tested but yielded suboptimal results [8].

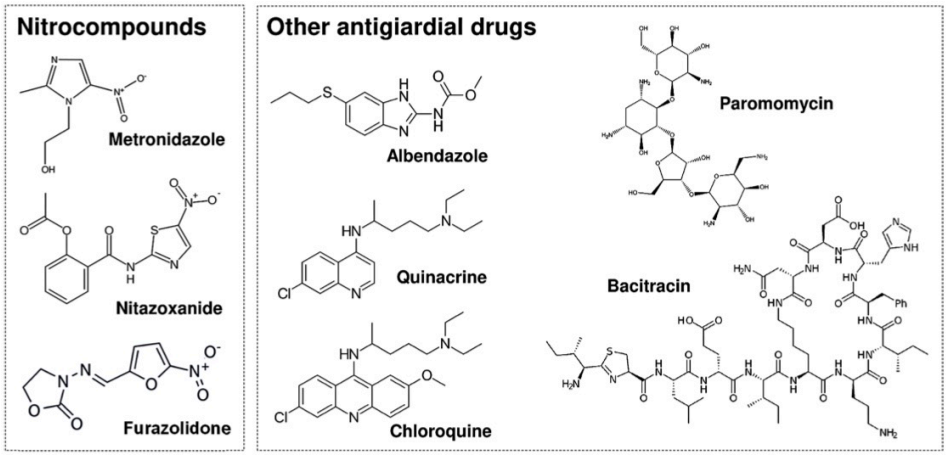

Figure 6. Current drug options for the treatment of giardiasis [287].

Newer 5-nitroimidazoles, such as tinidazole, a metronidazole derivative in use in Europe since 1969 and FDA-approved in 2004 [294], have also been developed. Secnidazole was approved in 2017 but not for giardiasis, while ornidazole remains unapproved. These drugs exhibit longer half-lives than metronidazole and have been evaluated for single-dose use [295]. Among them, tinidazole has been the most extensively studied. A literature review encompassing 60 randomized controlled trials concluded that tinidazole is more effective than metronidazole or albendazole [295], positioning it as a potential drug of choice [8].

Benzimidazole derivatives exert their effects by binding to β-tubulin, leading to irreversible damage to the parasite [296]. While mebendazole has generally produced suboptimal results, albendazole has demonstrated excellent efficacy when administered as a 5-day course [8].

Albendazole, which has an oral absorption rate of 1% to 5%, is metabolized into its active form, albendazole sulfoxide [297]. Some studies, including meta-analyses, suggest that albendazole is better tolerated than nitroimidazoles [298]. However, due to its embryotoxicity in animal studies, albendazole should be avoided during pregnancy [8].

Quinacrine was the first widely used drug for giardiasis and remained the preferred treatment for many years. It has a long half-life (5 to 14 days) and

is well absorbed from the gastrointestinal tract. Clinical studies report cure rates ranging from 77% to 100%, and it may reduce cyst viability [8,9]. However, quinacrine is associated with notable side effects, including a bitter taste, frequent vomiting, dizziness, and confusion. Additionally, it requires three daily doses over 5 to 7 days, which may complicate patient compliance [8]. Due to these adverse effects, quinacrine is generally considered a third-line treatment option [287].

Paromomycin, an aminoglycoside, is moderately effective and often used during the first trimester of pregnancy due to limited safety data for nitroimidazoles in this period [289]. However, given its lower efficacy, a more effective treatment should be prioritized for individuals with malnutrition or significant weight loss, even in the first trimester. Additionally, the antimalarial drug chloroquine has demonstrated efficacy comparable to nitroimidazoles in children [8,299].

Furazolidone is a nitrofuran derivative that has a similar efficacy to metronidazole (cure rate 80-96%) when used as initial therapy [300]; it damages the protozoan through producing superoxide radicals that damage DNA and the ability to differentiate into cyst [287,301].

Nitazoxanide is a nitro heterocyclic compound effective against *Cryptosporidium* spp. and *Giardia* [302]. After gastrointestinal absorption, it is rapidly converted to the active metabolite tizoxanide, rendering nitazoxanide undetectable in serum [8]. Its mechanism is proposed to involve noncompetitive inhibition of PFOR [303,304]. Administered as a 3-day course, nitazoxanide is well-tolerated, but its average efficacy of 71% is significantly lower than that of nitroimidazoles or albendazole [295].

In recent years, treatment failures with metronidazole have been reported in 10-20% of patients [305,306], raising concerns about drug resistance, particularly to nitroimidazoles [287]. While *in vitro* resistance to metronidazole has been documented [307], confirming resistant isolates requires isolating the parasite and demonstrating resistance *in vitro*, a process complicated by the difficulty of culturing *Giardia* and the lack of genetic markers for resistance [8]. Consequently, treatment-refractory is a more accurate term, though some studies suggest true resistance [308]. However, the increase in treatment failure from 15% to 40% between 2008 and 2013 among travelers returning to England suggests a true

development of resistance [309], although it is not yet know how many of these treatment failures are linked to actual resistance [8].
Regarding animals, fenbendazole and metronidazole are commonly used in most European countries to treat giardiasis in dogs and cats. The recommended dosage for fenbendazole is 50 mg/kg once daily for 3-7 days, while metronidazole is administered at 25 mg/kg twice daily for 5 days [310] (**Table 3**).

Molecules	**Dosage**
Fenbendazole*	50 mg/kg *per os sid* for 3-7 days
Metronidazole*	25 mg/kg *per os bid* for 5 days
Febantel, pyrantel, praziquantel	15.0 mg/kg for febantel, 14.4 mg/kg for pyrantel, and 5.0 mg/kg for praziquantel *per os sid* for 3 days
Fenbendazole + Metronidazole	50 mg/kg *per os sid* for 3-7 days + 25 mg/kg *per os bid* for 5 days
Metronidazole + Silymarin	25 mg/kg *per os bid* for 14 days + 3,5 mg/kg *per os sid* for 14 days
Ronidazole	30-50 mg/kg *per os sid* for 7 days

Table 3. Molecules and therapeutic regimens usable in the treatment of canine giardiasis [319]. * = molecule present in formulations registered in Italy for the treatment of giardiasis.

However, chemotherapy doesn't ensure pathogen elimination and persistent infections or reinfections are frequent [311]. Moreover, as for humans, metronidazole has been associated with strong adverse effects (i.e. anorexia, nausea, vomiting, diarrhoea weakness, hepatotoxicity, neutropenia) in pets [312,313]. Neurotoxicity may also occur because of chronic treatment or high acute doses and clinical signs include disorientation, ataxia, blindness, conscious proprioceptive deficits, hypermetria, tremors and nystagmus [314,315]. An alternative option in dogs' treatment (off-label use) is the combination of febantel (a pro-drug metabolized in vivo to fenbendazole), pyrantel, and praziquantel at the doses of 15.0 mg/kg for febantel, 14.4 mg/kg for pyrantel, and 5.0 mg/kg for praziquantel administered once a day for 3 days [310]. Other studies reported the efficacy of ronidazole, nitazoxanide, and secnidazole in

reducing *Giardia* cysts shed by infected dogs [316,317]. Moreover, other treatment options include a combination of fenbendazole and metronidazole, or metronidazole and silymarin [310,318] (**Table 3**).

It is not recommended to treat carriers that don't exhibit clinical signs, except for the cases in which the animals are at high risk for zoonotic transmission (contact with kids or immunocompromised subjects) or with other animals that may become infected by the sharing of the environment. Although there have been no reported cases of drug resistance in the veterinary field, a recent study reported a lack of efficacy against *G. duodenalis* in dogs [320]. Currently, no drugs are licensed specifically for treating *Giardia* infections in ruminants, but anthelmintic agents like albendazole and fenbendazole have been successfully employed [185,273]. The treatment of livestock remains a contentious issue due to the high prevalence of infection among these animals and the substantial environmental contamination with cysts, which makes reinfection highly probable [134,185].

In dogs, as in humans, the microbiota evolves rapidly in the first few months of life but remains stable in adults, although it is influenced by factors such as diet, weight, genetics, and other variables. *In vitro* and *in vivo* studies have shown that several species of lactobacilli, including *Lactobacillus johnsonii*, a species most isolated in pre-weaning puppies, can exert cytostatic and/or cytotoxic effects on trophozoites and are negatively correlated with *G. intestinalis* [321]. Allain *et al.* [201] demonstrated that *L. johnsonii* has anti-*Giardia* activity through the production of bile salt hydrolases *in vitro* and *in vivo* in the murine model. Infected mice, in fact, in numerous studies showed altered microbiota and a compromised epithelial barrier in the presence of this protozoan: it is hypothesized that these alterations could lead to changes in the canine model of the disease [322]. Regarding changes in the intestinal microbiota during *G. intestinalis* infections, we know that in humans this protozoan increases the presence of bacteria such as *Escherichia coli* and *Enterococcus* spp.; in dogs, however, most studies concerning the impact of *G. intestinalis* on the microbiota have focused on experimentally infected subjects, and therefore little is known about the intestinal microbiota during natural infections, especially in asymptomatic subjects [321]. Furthermore, during giardiasis in dogs, an increase in pro-inflammatory bacteria (such as *Prevotella*,

Suterella, *Veillonellaceae*, and *Ruminococcaceae*) has been observed, responsible for an increase in fecal calprotectin levels, a protein complex used as a marker of inflammation since it reflects the turnover of phagocytes, which normally tends to decrease with age in healthy animals [323]. All of these bacteria are also correlated with chronic gastrointestinal diseases such as Irritable Bowel Syndrome (IBS) in humans and Inflammatory Bowel Disease (IBD) in dogs. Furthermore, there is ongoing debate within the scientific community about whether *Giardia* acts as a primary agent or rather as an opportunistic pathogen capable of exerting its virulence in the presence of dysbiosis. This suggests that the preservation and restoration of intestinal eubiosis may play a key role in controlling the disease, potentially reducing or eliminating the need for pharmacological treatments. In the last 20 years, multiple studies have investigated the anti-*Giardia* properties of probiotic strains as an alternative strategy for the prevention and treatment of giardiasis, suggesting the use of probiotics and/or prebiotics to restore the intestinal microbiota and/or enhance the immune response. The probiotics most used in giardiasis are *Lactobacillus* and *Bifidobacterium*, which promote the production of secretory IgA through the expression of TGF-β, IL10, and IL6, as well as the Ig receptors on the surface of epithelial cells [324]. In the case of *Lactobacillus*, they share the same ecological niches with *G. intestinalis* in the proximal small intestine [323]. Reports on the *in vivo* effects of *Lactobacillus johnsonii*, *Lactobacillus rhamnosus*, and *Lactobacillus casei* in mice and gerbils have shown that these probiotics can significantly reduce morpho-functional alterations of the intestinal epithelium, thus reducing the duration and severity of the *Giardia* infection and inhibiting trophozoite activity[324]. *Lactobacillus acidophilus* and *Lactobacillus plantarum*, on the other hand, reduce both *in vivo* and *in vitro* the adhesion capacity of the trophozoite. Additionally, it has been shown that kefir, a fermented milk from ruminants, can protect mice from *Giardia* infection by reducing the infection and promoting the activation of various mechanisms of humoral and cell-mediated immunity [325]. Other microorganisms with anti-*Giardia* properties include *Enterococcus faecium* SF68, *Saccharomyces boulardii*, *Saccharomyces cerevisiae*, and *Candida* spp., as they stimulate the production of IgA and IgG and induce an inflammatory response [201,326].

1.5.2 Prevention and control

Handwashing plays a crucial role in reducing the risk of *Giardia* infection and prevents its transmission, particularly in cases of person-to-person, zoonotic, or foodborne spread. Groups at higher risk of contracting and transmitting giardiasis through these routes include children and staff in daycare centers, travelers, animal handlers, veterinarians, visitors to petting zoos, and food handlers [134]. It is vital to exclude symptomatic individuals from activities such as daycare participation, swimming, and food handling whenever possible, as they pose a significant risk of transmitting the infection. Public health education and training programs focusing on personal and food hygiene are essential for raising awareness and mitigating the risk of giardiasis and other enteric pathogens [134].

Controlling transmission among animals poses unique challenges: for dogs and cats, preventive measures include bathing, cleaning and disinfecting environments, and restricting access to untreated water and faecally contaminated areas (**Table 4**). Special attention should be given to kennels and shelters, where infections are more prevalent, and animal-to-animal transmission risk is higher [327]. In ruminants, where longitudinal studies often report 100% cumulative prevalence [185], improved husbandry and management practices are essential to reduce environmental cyst contamination and infection spread. Key practices include regular cleaning and disinfection, prompt removal of feces from animal housing, using single-cow calving areas, and ensuring neonates have access to colostrum [185].

For waterborne transmission, a multi-barrier approach is recommended; this includes restricting human and animal access to watersheds and reservoirs and employing treatment methods such as flocculation, filtration, and disinfection [133]. Although chlorine treatment reduces bacterial pathogens, it is less effective against the cysts of *Giardia*. Filtration methods commonly used in water treatment are effective in removing cysts, while advanced technologies such as ultraviolet light, ozone, and irradiation can inactivate parasites [134]. However, these methods are often costly and unavailable in smaller communities or developing regions. If contamination is suspected, drinking bottled water or boiling tap water for at least one minute can serve as emergency precautions [134].

Since fresh produce is frequently consumed raw without further processing, implementing control measures to minimize contamination risk or eliminate parasites is critical for consumer safety. Pre-harvest measures include using properly treated water, monitoring farm worker health and hygiene, improving on-farm sanitation, and preventing livestock and other animals from accessing cultivations and surface water [140,141]. Post-harvest measures prioritize using treated water for washing and processing produce, maintaining hygiene standards for hands and equipment, and enforcing good personal hygiene practices among food handlers [134].

The application of chemical and physical disinfectants to foods, as well as to working surfaces and equipment, should be considered as potential strategies to prevent foodborne transmission of protozoan parasites [141]. At the level of food handlers and consumers, maintaining good personal hygiene, particularly regular handwashing, is essential to minimize the risk of contaminating food with protozoan parasites. While specific experimental data on *Giardia* remain limited, interventions such as cooking and freezing can act as critical barriers to foodborne transmission. However, these methods are not universally applicable since many fruits and vegetables are consumed raw [134]. Lastly, travelers to regions with higher giardiasis prevalence, especially in developing countries, should be educated through travel clinics and public health campaigns to exercise caution in their food and beverage choices and to prioritize personal hygiene practices [272].

Disinfectant	Target	Dosage	References
4-chlorine-M-cresol	Floors, walls and installations	3% dilution with water, applying approximately 0.4 L/m^2	[316]
Quaternary ammonium disinfectant	Floors (it can inactivate *Giardia* cyst)	Follow the manufacturer's instructions	[328,329]
4% chlorhexidine digluconate	Fur	Shampoo left for 5–7 min twice a day	[316]

Table 4. List of disinfectants with proven activity against *Giardia duodenalis*; however, no disinfectants are registered to kill *Giardia* cysts.

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Chapter 2: Plant-based natural treatments for *Giardia duodenalis* infection

Since no effective vaccines are currently available to prevent *Giardia* infection, chemotherapy with synthetic drugs is currently the most reliable option for treating giardiasis in humans as well as in animals [1]. However, several studies have highlighted limitations associated with these synthetic drugs, including treatment-refractory cases and potential side effects (e.g., nausea, mild headache, dizziness, a metallic taste in the mouth, yellowing of the skin, and elevated liver enzymes) [1–3]. Recent efforts have focused on identifying new alternative antigiardial agents that provide enhanced efficacy and reduced toxicity. Historically, medicinal plants and their derivatives have played a significant role in promoting health and managing various diseases, including chronic conditions, due to their bioactive properties, low toxicity, and reduced risk of resistance development compared to synthetic drugs. Moreover, herbal medicines have demonstrated effectiveness in treating a wide range of bacterial, viral, fungal, and parasitic infections.

These natural products are commonly prepared as tinctures, syrups, teas, infusions, extracts, or powders [4]. Plant extracts, which are rich in active metabolites obtained from various plant parts, are frequently employed in scientific research. To prepare extracts for *in vitro* and *in vivo* studies, plant components (e.g., stems, roots, leaves, and seeds) undergo processes including maceration, separation, purification, and fractionation. These steps involve incubation with volatile solvents (e.g., hexane, dichloromethane, chloroform, acetone, methanol, or ethanol) to isolate bioactive compounds [5]. Notably, extracts often contain high concentrations of bioactive compounds, comparable to those found in infusions and teas, making them particularly valuable for research purposes. The present chapter reviews the existing literature on the use of herbal remedies for the treatment of giardiasis.

2.1 In vitro and in vivo assays

A total of 56 plant species from 21 families have shown anti-*Giardia* activity through *in vitro* and *in vivo* assays [4] (**Table 5)**.

Family	Scientific name	Part plant used	Preparation mode	Parasite form	References
Adoxaceae	*Sambucus ebulus*	Fruit	Aqueous extract	Cyst	[6]
Anacardiaceae	*Mangifera indica*	Aerial parts	Aqueous extract	Trophozoite	[7]
Amaryllidaceae	*Allium paradoxum*°	Leaves	Hydroalcoholic extract	Cyst	[8]
	*Allium sativum**	Aerial parts; bulb	Essential oil; hydroalcholic extract; chloroform extract	Both	[9–11]
Apiaceae	*Carum copticum*	Leaves	Aqueous extract, essential oil	Cyst	[12]
	Cuminum cyminum	Aerial parts	Aqueous extract	Trophozoite	[7]
	Echinophora cinerea	Aerial parts	Aqueous extract	Cyst	[13]
	Ferula assa-foetida	Aerial parts	Aqueous and ethanolic extracts; essential oil	Cyst	[14,15]
	Heracleum glabrescens	Grain	Methanolic extract	Cyst	[16]
Asparagaceae	*Yucca baccata*°	Stem	Aqueous extract	Trophozoite	[17]
Asteraceae	*Achillea santolina*	Aerial parts	Aqueous extract	Trophozoite	[7]
	Ageratum conyzoides	Flower leaf	Essential oil; hydroalcholic extract	Trophozoite	[18]
	Artemisia annua	Aerial parts; leaves	Hydroalcholic extract; chloroformic extract	Both	[19–21]
	Artemisia campestris	Aerial parts	Aqueous extract	Trophozoite	[7]

Family	Scientific name	Part plant used	Preparation mode	Parasite form	References
	Artemisia siberi	Aerial parts	Essential oil	Both	[10]
	*Pulicaria undulata**	Aerial parts	Aqueous extract	Cyst	[22]
	Tanacetum parthenium	Aerial parts	Chloroformic extract	Both	[19]
Chenopodiaceae	*Chenopodium botrys*	Aerial parts; seed	Essential oil; aqueous and alcoholic extracts	Cyst	[9,23]
Cistaceae	*Helianthemum glomeratum°*	Flower	Methanolic extract	Trophozoite	[24]
Combretaceae	*Terminalia ferdinandiana*	Fruit	Methanolic, aqueous, ethyl acetate, chloroform and hexane extracts	Trophozoite	[25]
Cucurbitaceae	*Cucurbita pepo L.*	Seed	Petroleum ether and methanolic extracts	Trophozoite	[26]
	Cucurbita maxima D.	Seed	Petroleum ether and methanolic extracts	Trophozoite	[26]
	Lagenaria siceraria	Seed	Petroleum ether and methanolic extracts	Trophozoite	[26]
Lamiaceae	*Lavandula angustifolia**	Aerial parts	Essential oil; aqueous extract	Both	[27,28]
	Lavandula intermedia	Aerial parts	Essential oil	Trophozoite	[28]
	Lavandula stoechas°	Aerial parts	Hydroalcoholic extract	Cyst	[28]
	Mentha longifolia	Leaves	Chloroformic extract	Trophozoite	[29]

Family	Scientific name	Part plant used	Preparation mode	Parasite form	References
	Mentha piperita	Leaves	Methanol, dichloro-methane and n-hexane extracts	Trophozoite	[30]
	Ocimum basilicum	Leaves	Petroleum ether, etyl acetate, methanol and aqueous extracts; chloroform extract	Trophozoite	[29,31]
	Origanum virens	Aerial parts	Essential oil	Trophozoite	[32]
	Origanum vulgare	Floweing aerial parts	Hydroalcholic extract	Cyst	[33]
	Rosmarinus officinalis	Leaves	Petroleum ether, etyl acetate, methanol and aqueous extracts	Trophozoite	[31]
	Satureja hortensis	Leaves	Methanolic extract	Cyst	[16]
	Satureja khuzestanica	Leaves	Hydroalcholic extract	Cyst	[9]
	Stachys lavandulifolia	Leaves	Aqueous and n-hexane extracts	Cyst	[34]
	Thymbra capitata	Aerial parts	Essential oil	Trophozoite	[35]
	Thymus vulgarize	Aerial parts	Essential oil	Cyst	[14]
	Thymus zygis	Aerial parts	Essential oil	Trophozoite	[32]
	Zataria multiflora	Aerial parts	Essential oil	Both	[10,14]
Myrtaceae	*Eucalyptus camaldulensis*	Aerial parts	Aqueous extract	Trophozoite	[7]
	Eucalyptus globulus	Aerial parts	Essential oil	Both	[10]
	Eucalyptus radiata	Leaves	Methanolic extract	Cyst	[16]

Family	Scientific name	Part plant used	Preparation mode	Parasite form	References
	Myrtus communis	Aerial parts	Essential oil	Cyst	[15]
	Syzygium aromaticum	Leaves	Aqueous extract	Trophozoite	[36]
Oleaceae	*Olea europaea*	Leaves	Hydroalcholic extract	Cyst	[9]
Poaceae	*Cymbopogon citratus**	Leaves	Aqueous extract	Cyst	[22]
Punicaceae	*Punica granatum°*	Peel	Methanolic extract	Cyst	[37]
Ranunculaceae	*Nigella sativa*	Seed	Ethanolic extract	Trophozoite	[38]
	Pulsatilla chinensis	Aerial parts	Ethyl acetate and aqueous extracts	Trophozoite	[39]
Rosaceae	*Rubus coriifolius°*	Fruits	Methanolic extract	Trophozoite	[27]
Rubiaceae	*Morinda royoc*	Root	Methanolic extract	Trophozoite	[40]
Rutaceae	*Citrus aurantifolia*	Peels	Hexane extract	Trophozoite	[41]
Verbenaceae	*Lippia beriandieri*	Aerial parts	Aqueous extract	Trophozoite	[42]
	Lippia graveolens	Aerial parts	Essential oil	Trophozoite	[32]
Zingiberaceae	*Curcuma longa**	Stem bark	Dichloromethane extract	Cyst	[43]
	*Zingiber officinale**	Root; rhizome	Aqueous extract; dichloromethane extract	Both	[35,43]

Table 5. Plants with antigiardial activity grouped by botanical families (in alphabetical order), detailing the type of extract used and the targeted parasite form (cysts, trophozoites, or both). Data includes plants tested *in vitro* and *in vivo* [4]. * = tested both *in vitro* and *in vivo*; ° = tested *in vivo* only.

In the **Adoxaceae** family, *Sambucus ebulus*, tested at doses of 1, 10, 50, and 100 mg/mL, demonstrated excellent activity against cysts using an aqueous extract of its fruits [6]. Within the **Anacardiaceae**, *Mangifera indica* was effective against trophozoites, reducing their viability by 75% with an aqueous extract of its aerial parts at a dose of 2000 mg/mL [7]. In the **Amaryllidaceae** family, *Allium paradoxum* was tested against *Giardia* cysts *in vivo* using Balb/c mice at doses of 20, 50, and 100 mg/mL. The 100 mg/mL dose effectively eliminated cysts, showing a significant effect compared to the control group ($p < 0.05$) [8]. *Allium sativum* (garlic) displayed broad activity against cysts and trophozoites. Hydroalcoholic extracts at 2 and 5 mg/mL produced a cyst fatality rate of 22.65 ± 10.47% [9]. Azadbakht *et al.* [10] reported significant parasiticidal effects of garlic essential oil at 0.1 and 0.2 μg/mL ($p < 0.05$). Moreover, a chloroform extract of *A. sativum* was tested *in vivo* using Balb/c mice at a dose of 80 mg/mL. The results indicated that *G. duodenalis* cysts were more sensitive to the garlic extract than *G. muris*. Although the infected mice in the test groups were not cured at doses of 20 and 40 mg/kg body weight, they became completely cyst-free with a dose of 80 mg/kg within three days [11]. In the **Apiaceae** family, *Carum copticum* exhibited dose- and time-dependent effects. After 60 minutes, the minimum inhibitory concentrations (MICs) of alcoholic extracts and essential oils were 100 mg/mL and 8 mg/mL, respectively. After 180 minutes, the MICs decreased to 75 mg/mL and 4 mg/mL [12]. *Cuminum cyminum* reduced trophozoite viability to 25% at 2000 mg/mL [7]. *Ferula assa-foetida* primarily affected cysts; ethanol extracts achieved 100% efficacy at 20 mg/mL within 4 hours, while aqueous extracts reached a maximum efficacy of 57.23% under similar conditions [14]. Another study highlighted the potential of *F. assa-foetida* extracts and essential oils for further development as herbal remedies against *G. duodenalis* [15]. Aqueous extracts of *Echinophora cinerea* at doses of 4 and 8 mg/mL caused the gradual destruction of cysts, with rates of 7.92 and 7.89 cysts/hour, respectively. The mean cyst destruction rate for 8 mg/mL of *E. cinerea* extract was 8.83 cysts/hour [13]. *Heracleum glabrescens* methanol extracts, tested at doses of 10, 100, and 200 mg/mL, demonstrated a 44% fatality rate at 200 mg/mL after 60 minutes [16]. In the **Asparagaceae** family, an aqueous extract of *Yucca baccata* was tested *in vivo* using Mongolian gerbils at doses of 24.4, 12.2,

and 6.1 mg/mL/day for three days against trophozoites. While the extracts reduced trophozoite counts in the duodenum segment, the reduction was not statistically significant. However, the highest extract concentration significantly reduced trophozoite counts in the proximal segment, showing an effect comparable to that of metronidazole [17]. Several plants in the **Asteraceae** family, such as *Achillea santolina*, *Artemisia campestris*, and *Ageratum conyzoides*, showed activity against trophozoites using aqueous or hydroalcoholic extracts, as well as essential oils. Notably, *A. santolina* and *A. campestris*, at 2000 mg/mL, completely eliminated *G. duodenalis* trophozoites [7]. Extracts from *A. conyzoides* (white–purple leaves and purple flowers) exhibited high activity ($IC_{50} \leq 100$ µg/mL) against trophozoites, with $IC_{50} \pm SD$ values of 45.67 ± 0.51 and 96.00 ± 0.46 µg/mL, respectively. The essential oils from these extracts had $IC_{50} \pm SD$ values of 35.00 ± 0.50 and 89.33 ± 0.41 µg/mL. Additionally, transmission electron microscopy (TEM) revealed degeneration of the flagella and ventral discs of *G. duodenalis* trophozoites following exposure to crude extracts [18]. Other species in the Asteraceae family, such as *Artemisia annua*, *Artemisia sieberi*, and *Tanacetum parthenium*, were effective against both cysts and trophozoites. Hydroalcoholic extracts of *A. annua* at 50 and 100 mg/mL caused significant cytotoxicity against cysts after 3 and 24 hours [21]. Chloroformic extracts of *A. annua* at 10 and 100 mg/mL reduced cyst viability by 96% and 99%, respectively, and completely eliminated trophozoites after 3 hours [19]. Similarly, varying concentrations (1, 10, 50, and 100 mg/mL) of *A. annua* chloroformic extracts achieved 100% elimination of cysts and trophozoites within 180 minutes [20]. Essential oils from *A. sieberi*, tested at concentrations of 0.001–0.2 µg/mL, demonstrated significant parasiticidal effects at 0.1 and 0.2 µg/mL ($p < 0.05$) [10]. The aqueous extracts of *Pulicaria undulata*, tested at a dose of 200 mg/kg, demonstrated effectiveness against cysts both *in vitro* and *in vivo*, suggesting their potential as natural therapeutic alternatives to metronidazole [22]. The chloroformic extract of *T. parthenium* at concentrations of 1 and 10 mg/mL killed 97% and 99% of cysts and completely eliminated trophozoites at 1 mg/mL within 3 hours. At 50 mg/mL, it achieved 100% elimination of trophozoites within 1 hour [19]. In the **Chenopodiaceae** family, *Chenopodium botrys* exhibited activity against cysts using aqueous, alcoholic, and essential oil extracts. The

essential oil at concentrations of 0.1 and 0.2 µg/mL showed significant parasiticidal effects ($p < 0.05$) [9]. In another study, the highest antigiardial effects of alcoholic and aqueous extracts of *C. botrys* were observed at 37 °C with 20 mg/mL, achieving 100% and 66.1% efficacy, respectively, 5 hours post-experiment [23]. *Helianthemum glomeratum* (family **Cistaceae**) was tested *in vivo* using CD-1 mice as a methanolic extract at doses of 1.25, 2.5, 5, 10, and 20 mg/kg against trophozoites. The effective dose (ED50) was determined to be 0.125 mg/kg. The extract showed efficacy comparable to that of metronidazole [24]. The growth-inhibitory potential of *Terminalia ferdinandiana* (family **Combretaceae**) fruit extracts against trophozoites was evaluated. Methanolic and aqueous extracts exhibited the strongest activity, with IC_{50} values of approximately 700 µg/ml and 140 µg/ml, respectively. Ethyl acetate and chloroform extracts also demonstrated inhibitory effects, albeit with lower potency. In contrast, the hexane extract showed no inhibitory activity against *G. duodenalis* [25]. In the **Cucurbitaceae** family, seeds from *Cucurbita pepo L.*, *Cucurbita maxima D.*, and *Lagenaria siceraria* showed activity against trophozoites when processed with petroleum ether and methanol. *C. pepo L.* petroleum ether extracts caused 100% mortality within 120 hours, with an IC_{50} of 60671.32 ppm at a concentration of 500 ppm [26]. *C. maxima D.* petroleum ether extracts caused 100% mortality within 48 hours, with an IC_{50} of 548.80 ppm at concentrations of 1000 and 500 ppm [26]. *L. siceraria* petroleum ether extracts, tested at doses of 1000, 500, and 250 ppm, achieved 100% mortality within 72 hours, with an IC_{50} of 95.65 ppm [26]. The most widely used medicinal plants against *G. duodenalis* infection belong to the **Lamiaceae** family. *Lavandula angustifolia* and *L. intermedia*, tested at concentrations of 0.1%, 0.5%, and 1%, demonstrated that low doses (≤1%) of their essential oils could eliminate trophozoites [28]. Moreover, *L. angustifolia* and *L. stoechas* were evaluated *in vivo* using Souri mice and Swiss albino mice, respectively. The results indicated that *L. angustifolia* has promising effects *in vivo* and could serve as a viable alternative for the treatment of giardiasis. Treatment with the extract of *L. stoechas* significantly reduced cyst excretion rates by 95.1%, 84.3%, and 77.7% following administration at doses of 400, 200, and 100 mg/mL over 10 days [27,28]. Regarding *Mentha longifolia*, chloroform extracts at a dose of 200 µg/mL inhibited more than 20% of trophozoites [29]. In contrast, aqueous

extracts of *Mentha piperita* showed no effect against trophozoites, with an IC_{50} > 100 µg/mL. However, the aqueous fraction exhibited moderate activity (IC_{50} = 45.5 µg/mL), while the dichloromethane fraction demonstrated the highest antigiardial activity (IC_{50} = 0.75 µg/mL) after 48 hours of incubation. Morphological and adhesion assays revealed that the dichloromethane fraction caused severe alterations to the parasite's plasma membrane and inhibited trophozoite adhesion [30]. *Ocimum basilicum* chloroform extracts showed strong activity against trophozoites, with an IC_{50} of 53.31 µg/mL [29]. Petroleum ether extracts (IC_{50} = 14.9 mg/mL), ethyl acetate extracts (IC_{50} = 25.4 mg/mL), and methanol extracts (IC_{50} = 33.8 mg/mL) from *O. basilicum* also exhibited potent activity with an overall IC_{50} of 2.383 mg/mL [31]. *Origanum virens*, tested at doses of 10–300 µg/mL, showed an IC_{50} of 85 µg/mL against trophozoites. Morphological changes included a rounded shape, irregular dorsal and ventral surfaces, the presence of membrane blebs, electrodense precipitates in the cytoplasm and nuclei, and internalization of flagella and ventral discs [32]. *Origanum vulgare*, tested at doses of 10, 100, and 200 mg/mL against cysts, exhibited antigiardial activity. Hydroalcoholic extracts at 200 mg/mL showed similar efficacy to metronidazole ($p > 0.05$) [33]. *Rosmarinus officinalis* petroleum ether extracts (IC_{50} = 4.382 mg/mL), ethyl acetate extracts (IC_{50} = 2.02 mg/mL), and methanol extracts (IC_{50} = 2.383 mg/mL) showed strong activity against trophozoites [31]. Methanol extracts of *Satureja hortensis* at 200 mg/mL achieved a fatality rate of 84.3% within 60 minutes [16]. Hydroalcoholic extracts of *Satureja khuzestanica* demonstrated a fatality rate of 32.52 ± 9.07% against cysts [9]. The aqueous extract of *Stachys lavandulifolia* at 100 mg/mL killed 93% of cysts after 6 hours, while the n-hexane extract at the same concentration achieved 100% efficacy within the same time frame. Both extracts exhibited dose-dependent antigiardial activity, with the n-hexane extract being more effective [34]. *Thymbra capitata* exhibited an IC_{50} of 71 µg/mL against trophozoites. Morphological changes observed included a rounded shape, irregular dorsal and ventral surfaces, the presence of membrane blebs, electrodense precipitates in the cytoplasm and nuclei, and internalization of flagella and ventral discs [35]. Studies on *Thymus vulgaris* suggested that essential oil is effective against cysts and have potential for the development of herbal remedies [14]. The essential oil of *Thymus zygis* exhibited an IC_{50} of 185 µg/mL, with

morphological changes similar to those described for *T. capitata* [32]. The essential oil of *Zataria multiflora* showed significant activity against trophozoites and cysts at concentrations of 0.1 and 0.2 µg/mL ($p < 0.05$) [10,14]. In the **Oleaceae** family, hydroalcoholic extracts of *Olea europaea*, tested at doses of 2 and 5 mg/mL against cysts, showed a maximum fatality rate of 37.90 ± 7.01% [9]. In the **Myrtaceae** family, several species have demonstrated activity against *G. duodenalis*. *Eucalyptus globulus*, *E. camaldulensis*, and *E. radiata* were effective against trophozoites, cysts, or both forms. The aqueous extract of *E. camaldulensis* at a dose of 2000 mg/mL completely eliminated trophozoites [7]. The essential oil of *E. globulus* showed significant parasiticidal effects at concentrations of 0.1 and 0.2 µg/mL ($p < 0.05$)) [10]. Methanol extracts of *E. radiata*, diluted to 200 mg/mL, achieved a fatality rate of 63.3% within 60 minutes [16]. Additionally, the essential oil of *Myrtus communis* showed beneficial effects against cysts [15]. Water extracts of *Syzygium aromaticum* displayed significant activity against trophozoites, with an IC_{50} of 0.755 mg/mL [36]. In the **Poaceae** family, *Cymbopogon citratus* was effective against cysts using aqueous extracts. Both *in vitro* and *in vivo* (balb/c mice) studies indicated its potential as a natural therapeutic alternative to metronidazole, with effective doses of 500 mg/mL [22]. The methanolic extract of *Punica granatum* (family **Punicaceae**) was tested *in vivo* on Swiss albino mice against cysts at a dose of 300 mg/kg/day for 30 days. The results revealed that the prevention rate in the experimental groups reached approximately 50% by the 10th day of treatment. Additionally, stool cyst counts showed a significant reduction in cyst shedding, with an approximately 75.6% decrease by day 20 post-infection [37]. The **Ranunculaceae** family, represented by *Nigella sativa* and *Pulsatilla chinensis*, showed activity against trophozoites. *N. sativa* achieved 95% mortality within 96 hours at a concentration of 500 µg/mL against trophozoites [38]. Water and ethyl acetate extracts of *P. chinensis* inhibited trophozoite adherence after 3 hours of incubation and killed nearly 50% of the parasite population in a time-dependent manner. Morphological changes included dissolved cytoplasm with large vacuoles, membrane blebs, breaks in flagella and ventral discs, and intracellular clearance, as observed by electron microscopy [39]. The methanolic extract of *Rubus coriifolius* (family **Rosaceae**) was tested *in vivo* on CD-1 mice at doses of 1.25, 2.5, 5, 10, and 20 mg/kg against

trophozoites. The extract demonstrated antigiardial activity, with an ED_{50} of 0.506 mg/kg [27]. The methanolic extract of *Morinda royoc* root (family **Rubiaceae**) exhibits antigiardial activity against trophozoites without affecting cell viability; the hexane fraction demonstrated strong activity (IC_{50} = 0.08 µg/mL) and contained an anthraquinone-type compound as its principal component [40]. In the **Rutaceae** family, *Citrus aurantifolia* was tested at doses of 1–10 mg/mL against trophozoites. Compounds such as 4-hexen-3-one, citral, and geraniol exhibited IC_{50} values of 34.2, 64.5, and 229.49 µg/mL, respectively, in axenic cultures after 24 hours. Comparatively, metronidazole was significantly more potent, as 4-hexen-3-one, citral, and geraniol were 66, 112, and 441 times less active, respectively [41]. In the **Verbenaceae** family, aqueous extracts of *Lippia berlandieri* achieved 90% mortality in trophozoites [42]. Essential oil from *Lippia graveolens* showed an IC_{50} value of 257 µg/mL. Morphological changes included rounded shapes, irregular dorsal and ventral surfaces, membrane blebs, and electrodense precipitates in the cytoplasm and nuclei, as well as internalized flagella and ventral discs [32]. In the **Zingiberaceae** family, *Curcuma longa* (curcumin) and *Zingiber officinale* (ginger) showed efficacy against both cysts and trophozoites using dichloromethane and aqueous extracts. For *C. longa*, a dichloromethane extract at 50 mg/mL achieved an 85% cyst mortality rate within 60 minutes. *In vivo* testing on Balb/c mice revealed that curcumin, administered at doses of 10 and 20 mg/kg/day, significantly reduced the excretion of dead cysts and intestinal trophozoites, with an 84.7% reduction observed at the 20 mg/kg/day dose [43]. Similarly, water extracts of *Z. officinale* were found to be as effective against trophozoites as nitazoxanide [35]. Additionally, a dichloromethane extract of *Z. officinale* demonstrated a 97% cyst mortality rate within 60 minutes at a concentration of 50 mg/mL. *In vivo*, ginger extract administered to Balb/c mice at doses of 10 and 20 mg/kg/day led to a complete reduction in the excretion of dead cysts and intestinal trophozoites at the 20 mg/kg/day dose, comparable to the efficacy of metronidazole at the same [43].This overview emphasizes the broad diversity of plants and preparations with potential antigiardial properties. The efficacy of these plants is attributed to their high concentrations of bioactive compounds, such as flavonoids, phenolic acids, and terpenoids. Flavonoids act by disrupting the integrity of the parasite's membrane,

inhibiting nucleic acid synthesis, and interfering with enzymatic activities. Terpenoids, on the other hand, compromise membrane stability and cellular functions, ultimately leading to parasite death. It is also noteworthy that the studies employed a variety of extraction methods, including aqueous extracts, essential oils, and hydroalcoholic solutions. Aqueous extracts are particularly favored for their ease of preparation and ability to preserve the activity of heat-sensitive compounds. Moreover, the review highlights that the aerial parts and leaves of plants were the most used components. These plant parts are preferred not only for their rich bioactive content but also for their sustainable harvesting practices, which minimize harm to the plants [4].

2.2 Clinical trials

Clinical trials investigating the efficacy of natural products against *G. duodenalis* represent a promising area of research aimed at identifying effective and safe therapeutic alternatives. Among the natural compounds tested in clinical studies there are extracts from *Tanacetum vulgare* [44], *Mentha crispa* [45], and *Anethum graveolens* [46]. The aims of these studies were to confirm the therapeutic potential of these extracts and to assess their tolerability and safety compared to conventional drugs. Preliminary findings suggest these plants hold promise as alternative treatments for *Giardia* infections, broadening the spectrum of natural therapeutic options. *Anethum graveolens* (Family **Apiaceae**), an annual herb cultivated in Iraq for both medicinal and culinary purposes, was tested to evaluate the efficacy of an aqueous extract of *A. graveolens* leaves in treating giardiasis compared to the standard drug metronidazole. A prospective, randomized clinical trial was conducted over six months (June–December 2013), involving 28 pediatric patients (aged 3–11 months) attending a private outpatient clinic in Baghdad. Participants were randomly divided into two groups: Group A (14 patients) received metronidazole (15 mg/kg) three times daily for five days, while Group B (14 patients) received 1 ml of an aqueous extract of *A. graveolens* three times daily for five days. Stool samples were collected at baseline, after five days, and 14 days post-treatment to assess efficacy. Results demonstrated a significant reduction in *G. duodenalis* incidence following treatment with the aqueous extract of *A. graveolens*, showing comparable efficacy to metronidazole. The study

concluded that a five-day regimen of the aqueous extract of *A. graveolens* is a safe, tolerable, and effective alternative treatment for pediatric giardiasis, offering symptom improvement similar to that of metronidazole [46].

A double-masked, placebo-controlled study was conducted to evaluate the effects of *Triticum vulgare* (Family **Poaceae**) germ (WG) dietary supplementation in 63 giardiasis patients in Montreal and Lima, including 25 asymptomatic subjects passing cysts and 38 symptomatic patients. Asymptomatic subjects received WG (2 g, three times daily) or placebo (cornstarch) for 10 days, followed by metronidazole (250 mg, three times daily) for 7 days. Symptomatic patients received metronidazole with either WG or placebo for 7 days. Stool samples were collected daily in Montreal or every other day in Lima during treatment and again on Day 35 for microscopic and coproantigen analysis, while subjects maintained a symptom diary for 10 days. In asymptomatic subjects, WG supplementation reduced cyst passage and coproantigen levels by approximately 50% compared to placebo ($p < 0.01$ and $p = 0.06$, respectively). In symptomatic patients, while metronidazole effectively decreased cyst passage and coproantigen levels in all cases, symptom resolution appeared faster with the addition of WG. The WG supplement was well-tolerated across all groups, suggesting that WG components, likely WGA, may influence the progression of giardiasis, either alone or as an adjunct to antiprotozoal therapy [44]. Lastly, through a randomized, open-label clinical trial with an active control, the aqueous extract of *Mentha crispa* (Family **Lamiaceae**) leaves was tested to evaluate its therapeutic efficacy in treating giardiasis. The research began with a cross-sectional study to identify subjects with giardiasis. From May 2005 to May 2007, coprology samples were collected from 1,622 patients for parasitological examinations. Ninety-six patients diagnosed with *G. duodenalis* were randomized into two groups: one treated with secnidazole (2 g; $n = 50$) and the other with *M. crispa* (2 g; $n = 46$). After 7 days, cure rates were assessed using enzyme immunoassay on fresh fecal samples. Results showed a significantly higher cure rate in the secnidazole group (84.0%) compared to the *M. crispa* group (47.83%; $p = 0.0002$); while *M. crispa* demonstrated some efficacy, its therapeutic effect at the dose used in this study was notably less effective than secnidazole. The study concludes that *M. crispa*

may have limited utility as a standalone treatment for giardiasis at the evaluated dosage [45].

Despite these promising outcomes, the adoption of plant-based therapies faces challenges. One major limitation is the lack of extensive clinical trials to confirm efficacy, safety, and optimal dosing in human populations. Furthermore, concerns about the potential toxicity of some plant extracts and the standardization of herbal preparations remain barriers to their widespread application [46]. In conclusion, medicinal plants offer a promising alternative or complementary approach to the treatment of giardiasis, with several advantages, including fewer side effects, a lower risk of resistance, and a broad range of mechanisms of action. By integrating traditional knowledge with modern pharmacological research, plant-based therapies could play a crucial role in addressing the global burden of giardiasis, particularly in regions with limited access to conventional drugs [46].

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Chapter 3: Testing natural compounds *in vitro* for their antigiardial activity

1. Introduction

Current treatments for giardiasis consist in prolonged medication regimens for both humans and animals. In humans, metronidazole (MTZ) is the first-line treatment, with a recovery rate exceeding 90% [1,2]. However, MTZ is associated with side effects, including headache, nausea, glossitis, neutropenia, and has been linked to carcinogenic, teratogenic, and embryotoxic effects [2,3]. Since giardiasis infections are often self-limiting, a conservative approach may be considered for mild cases. However, active treatment is recommended for symptomatic giardiasis to alleviate symptoms, reduces the duration of illness and potential transmission, and prevent complications [4]. In Europe, fenbendazole (FBZ) (50 mg/kg once daily for 3-7 days) and MTZ (50 mg/kg once daily for 3-7 days) are commonly used to treat giardiasis in dogs and cats [5]. Despite chemotherapy, complete eradication of the pathogen is not guaranteed, and reinfections or persistent infections are frequent [6]. As in humans, MTZ in pets can cause side effects, including anorexia, nausea, vomiting, diarrhea, weakness, hepatotoxicity, and neutropenia [7,8]. Neurotoxicity is also a concern, particularly with long-term or high-dose treatments [9,10]. Treatment is not recommended for asymptomatic carriers unless the animals are at high risk of zoonotic transmission (e.g., exposure to children or immunocompromised individuals) or may infect other animals in shared environments. Recent reports indicate treatment failures with MTZ in 10-20% of human cases [11,12] and although drug resistance has not been reported in veterinary cases, a recent study found MTZ ineffective against *G. duodenalis* in dogs [13]. This emerging resistance, coupled with concerns about carcinogenic potential of MTZ, underscores the need for alternative treatments. Throughout history, plant-derived medicines have been utilized for a wide range of diseases, including parasitic infections [14,15]. The antiprotozoal properties of plants have been validated through the development of drugs isolated from plant species, such as emetine, quinine, and artemisinin [2,16–18]. Despite the complexity and limited availability of many novel plant compounds, they remain a promising source of therapeutic alternatives. A comprehensive review by Newman and Cragg [19]

of 1,562 new drugs approved between 1981 and 2014 found that only 27% were chemically synthesized, while the majority were either natural products or synthetic derivatives based on natural compounds [20].

The present study aimed to evaluate the potential of four natural plant extracts (*Morinda citrifolia*, *Panax ginseng*, *Tabebuia avellanedae*, and *Zingiber officinale*) and two isolated plant bioactive compounds (β-lapachone and 6-gingerol) against *G. duodenalis* trophozoites, with the goal of assessing their potential as allies in the fight against giardiasis. *M. citrifolia* and *P. ginseng* were selected due to their demonstrated efficacy *in vitro* and *in vivo* against other protozoan parasites, such as *Leishmania* spp. [21] and *Plasmodium* spp. [22], and not having been previously tested against *G. duodenalis*. The anti-protozoal properties of *T. avellanedae* have been well-documented in studies against *Trypanosoma cruzi*, *Leishmania* spp., and *Plasmodium falciparum* [23–26]. Additionally, previous research has shown that β-lapachone induces cell death in *G. duodenalis* [27]. Although *Z. officinale* has been previously tested against *Giardia* cysts [28,29], has been chosen to investigate its effects on *G. duodenalis* trophozoites to determine whether it is also effective in this form, and to assess whether 6-gingerol, one of its bioactive compounds, contributes to its efficacy against giardiasis.

2. Materials and methods

2.1 Natural dry extracts and chemical compounds

The natural and chemical compounds used in the present work are listed in Table 1 and Table 2. *Morinda citrifolia* L., *Panax ginseng*, *Tabebuia avellanedae*, and *Zingiber officinale* Rosc. dry extracts were provided by Deakos SRL (Italy). β-lapachone ($C_{15}H_{14}O_3$), 6-gingerol ($C_{17}H_{26}O_4$), and MTZ ($C_6H_9N_3O_3$) were purchased from Merck (Sigma-Aldrich, Merck Life Science S.r.l., Milan, Italy). For the stock solutions, all natural and chemical compounds were dissolved in dimethyl sulfoxide (DMSO) and maintained at a concentration of 10 mM for β-lapachone, 6-gingerol, and MTZ, while each dry extract was dissolved to a concentration of 100 mg/ml. All compounds were stored in dark and frozen conditions.

Botanical species	Family	Part used	Preparation mode
Morinda citrifolia L.	Rubiaceae	Fruit juice	Lyophilization (dry extract)
Panax ginseng	Araliaceae	Root	Ethanol extract (dry extract)
Tabebuia avellanedae	Bignoniaceae	Bark	Hydroalcoholic extract (dry extract)
Zingiber officinale Rosc.	Zingiberaceae	Rhizome	Supercritical carbon dioxide (dry extract)

Table 1. Plant names and their parts used for the extracts tested for antigiardial activity.

Active compound	Compound class	Plant family	Plant species
β-lapachone	Naphthoquinones	Bignoniaceae	*Tabebuia avellanedae*
6-gingerol	Gingerols	Zingiberaceae	*Zingiber officinale*

Table 2. Bioactive compounds tested in this study with their class, plant family and plant species.

2.2 Parasite isolates and cultivation

Trophozoites of *G. duodenalis* WBC6 (ATCC-50803) (Assemblage AI) and GS/M (ATCC-50581) (Assemblage B) were axenically grown at 37 °C in flat-sided 10 mL screw-cap tubes (Nunclon; Thermo Fisher Scientific, Waltham, MA, USA) containing filter-sterilized Keister's modified TYI-S-33 medium, supplemented with 10% adult bovine serum (Gibco, Grand Island, NY), 100 mg/mL streptomycin, 100 U/mL penicillin (Capricorn, Frederick, MD), and 0.05% bovine bile (Sigma-Aldrich, St. Louis, MO) [30]. The tubes were filled with at least 10 mL of culture medium to ensure low-oxygen conditions. Confluent cultures were split two to three times per week by placing the tubes on ice for at least 15 minutes to detach the trophozoites, which were subsequently cultured after dilution (1:10 or 1:100) with fresh medium.

2.3 In vitro susceptibility assays

The drug susceptibility of *G. duodenalis* trophozoites was assessed using a modified bioluminescent ATP content assay, as previously described by Chen et al. [31]. The assays were performed in 96-well microplates with flat, clear bottoms. Trophozoites from a log-phase culture were harvested by chilling the culture on ice for at least 15 minutes and

then counted using a hemocytometer (Kova™, Thermo Fisher Scientific, Waltham, MA, USA). Trophozoites were seeded at 0.5×10^5 per well in 200 µl of modified TYI-S-33 medium. Compounds were prepared as two-fold serial dilutions in DMSO at 100X concentration and added to each well. Metronidazole and medium with DMSO were used as controls. The final DMSO concentration did not exceed 1%, which has been shown not to inhibit *G. duodenalis* growth [32]. The cells were incubated at 37°C for 48 hours under anaerobic conditions by placing the microplates in Aerogen Oxoid jars with appropriate reaction bags (Oxoid #AN0025). After incubation, trophozoite viability was measured using the bioluminescent ATP content assay (CellTiter-Glo™ 2.0, Promega Italia, Milano, Italy), following the manufacturer's instructions. Each experiment was performed in triplicate, with at least three biological replicates. The half-maximal inhibitory concentrations (IC_{50}) were calculated using nonlinear regression curve analysis in GraphPad Prism version 9 (GraphPad Software, San Diego, USA).

3. Results and discussion

In the present study we evaluated the *in vitro* activity of natural dry extracts from *M. citrifolia* L., *P. ginseng*, *T. avellanedae* and *Z. officinale* Rosc., along with two isolated bioactive compounds (6- gingerol from *Z. officinale* and β-lapachone from *T. avellanedae*) against *G. duodenalis* trophozoites. The calculated IC_{50} values of the dry extracts and chemical compounds are shown in Table 3. The hydroalcoholic extract of *T. avellanedae* demonstrated significant activity against *G. duodenalis* trophozoites. Furthermore, one of the bioactive compounds of *T. avellanedae*, β-lapachone, exhibited potent antigiardial activity, consistent with findings from a previous study [27]. Both β-lapachone and MTZ exhibited similar IC_{50} values across the two *G. duodenalis* isolates tested. Notably, β-lapachone proved to be more potent than MTZ, showing consistently lower 48-hour IC_{50} values for each isolate, with tight confidence intervals (CI) for all measurements, indicating high reliability of the data. In contrast, the other dry extracts *Morinda citrifolia* L., *Panax ginseng*, and *Zingiber officinale* Rosc. along with its bioactive compound 6-gingerol, showed no activity against *G. duodenalis* trophozoites.

Natural or chemical compound	WBC6	GS/M
Morinda citrifolia L. (mg/ml) IC_{50} (95% CI)	N.A	N.A
Panax ginseng (mg/ml) IC_{50} (95% CI)	N.A	N.A
Tabebuia avellanedae (mg/ml) IC_{50} (95% CI)	1.5 (1.45 - 1.55)	1.28 (1.24 – 1.33)
Zingiber officinale Rosc. (mg/ml) IC_{50} (95% CI)	N.A	N.A
β-lapachone (μM) IC_{50} (95% CI)	6.2 (5.41 – 7.04)	5.9 (5.26 – 6.71)
6-gingerol (μM) IC_{50} (95% CI)	N.A	N.A
Metronidazole (μM) IC_{50} (95% CI)	6.77 (6.11 – 7.5)	7.77 (7.23 – 8.36)

Table 3. The IC_{50} values of plant extracts and chemical compounds incubated for 48 hours with *G. intestinalis* trophozoites *in vitro*. N.A. = not applicable

It is worth noting that the antiprotozoal activity of some plants selected for the present study has already been reported. However, we chose to evaluate these species because their antiprotozoal properties were demonstrated using different parasites, plant species, plant parts to produce the extract or different types of extract. For example, while *M. citrifolia* L. has never been tested against *G. duodenalis*, a clinical trial assessed the efficacy of a topical unguent containing *M. citrifolia* L. stem extract for treating cutaneous leishmaniasis. The results showed an excellent response in 50% of the 40 patients treated and significant improvement in 30% [33]. In another study, the fruit juice of *M. citrifolia* L. was evaluated *in vivo* against *Leishmania infantum*, yelding positive results [21]. Furthermore, a *Morinda royoc* root extract and its fractions exhibited antigiardial activity without affecting cell viability [34]. In contrast, *P. ginseng* has never been evaluated against *G. duodenalis in vitro*, nor against other protozoa. However, ginsenosides and ginseng polysaccharides have demonstrated *in vivo* anti-*Plasmodium yoelii* activity [22]. Consequently, we decided to test it for the first time against *Giardia*, considering its numerous beneficial properties, such as significant effects on the central nervous system and metabolic, infectious, and neoplastic diseases [35]. Additionally, toxicological studies have demonstrated the good safety profile of ginseng [35].

A *Z. officinale* extract was tested *in vitro* against trophozoites of *G. duodenalis,* and the treatment significantly reduced the number of the parasites after 24 and 48 hours [28]. Dyab *et al.* [29] evaluated dichloromethane extracts of *Z. officinale* both *in vitro* and *in vivo* against *G. duodenalis* cyst. The *in vitro* tests revealed a significant effect on cyst viability, in a dose- and time-dependent manner. *In vivo*, the treatment significantly reduced fecal cyst and intestinal trophozoite counts in infected mice. Furthermore, *Z. officinale* has demonstrated *in vitro* efficacy against *Blastocystis* spp. [36]. However, in *in vitro* tests conducted, neither *M. citrifolia* L., *P. ginseng*, nor *Z. officinale* exhibited any antigiardial activity. As for *M. citrifolia* and *Z. officinale*, this lack of activity may be attributed to differences in species or extract types, as various species may contain bioactive compounds in different concentrations, which can influence their effectiveness against pathogens. Notably, 6-gingerol, a major compound in *Z. officinale*, had never been tested against *G. duodenalis* before and was found to be ineffective in the present *in vitro* tests. The results obtained suggest that 6-gingerol is not the bioactive compound responsible for the antigiardial activity observed in other ginger extracts.

Tabebuia avellanedae is a tree with significant ethnopharmacological uses. Its inner bark, leaves, and other parts have been employed in traditional medicine since the Inca civilization to treat various conditions, including cancer [37], obesity [38], depression [39], infections (viral, fungal, and bacterial) [40], as well as arthritis and colitis [41]. Known for its anti-cancer properties, the inner bark of *T. avellanedae* contains naphthoquinones compounds (lapachol, α-lapachone, and β-lapachone), which are primarily responsible for its medicinal effects. These quinones generate reactive oxygen species (ROS) through electron transfer, leading to oxidative stress and cellular damage, which contribute to their anti-cancer and antimicrobial effects [42–45]. While lapachol initially showed tumor-inhibitory effects, it was found unsuitable for clinical use due to toxicity and poor bioavailability [46,47]. However, both α-lapachone and β-lapachone have demonstrated potent activity against various cancers, including drug-resistant ones [48–51]. These compounds work by inhibiting topoisomerase I and interacting with quinone oxidoreductase (NQO1), inducing a cycle that causes apoptosis and metabolic stress [42].

The anti-protozoan properties of *T. avellanedae* have been evaluated against *Trypanosoma cruzi*, *Leishmania* spp., and *Plasmodium falciparum* through both *in vivo* and *in vitro* studies [23–26]. Notably, one study investigated the effect of β-lapachone on *G. duodenalis* and found that β-lapachone induces cell death and morphological alterations in *G. duodenalis in vitro* while promoting the expression of encystation markers [27]. Microscopic analysis revealed signs of apoptosis, including cell shrinkage, chromatin condensation, membrane blebbing, and vacuolization, as well as features of autophagy, such as myelinic figures in vacuoles. Drug treatment disrupted lipid rafts, which co-localized with membrane blebbing, and triggered encystation [27]. Furthermore, Park *et al.* [52,53] found that *T. avellanedae* extracts selectively inhibit harmful bacteria like *Clostridium* spp. and *Helicobacter pylori*, while sparing beneficial gut flora.

In conclusion, the present results highlight the *in vitro* efficacy of *T. avellanedae* against *G. duodenalis* trophozoites, particularly β-lapachone, which showed greater efficacy than MTZ. This underscores its potential therapeutic applications and suggests that further research is needed to assess not only its efficacy but also its safety.

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Chapter 4: Selective activity of *Tabebuia avellanedae* against *Giardia duodenalis* infecting organoid-derived human gastrointestinal epithelia

Giulia Rigamonti[a]*, Fabrizia Veronesi[a]*, Elisabetta Chiaradia[a], Petra Gosten-Heinrich[b], Antonia Müller[b], Leonardo Brustenga[a], Stefano de Angelis[c], Alessia Tognoloni[a], Riccardo De Santo[d], Christian Klotz[b*#], Marco Lalle[e*#]

[a]Department of Veterinary Medicine, University of Perugia, via San Costanzo 4, Perugia, Italy.
[b]Department of Infectious Diseases, Unit 16 Mycotic and Parasitic Agents and Mycobacteria, Robert Koch-Institute, Seestrasse 20, Berlin, Germany.
[c]Deakos SRL, Corso Nazionale 169, La Spezia, Italy.
[d]Department of Chemistry, Sapienza University, P.le Aldo Moro, 5. 00185, Rome, Italy.
[e]Department of Infectious Diseases, Unit of Foodborne and Neglected Parasitic Diseases, Istituto Superiore di Sanità, viale Regina Elena 299, Rome, Italy.

[#]Corresponding Authors
marco.lalle@iss.it; KlotzC@rki.de.
*These authors have equally contributed at the work

Abstract

Giardia duodenalis is a widespread intestinal protozoan that affects mammals, including humans. Symptoms can range from being subclinical to causing severe abdominal pain and diarrhoea. Giardiasis often requires repeated treatment with synthetic drugs like metronidazole. In recent years, treatment failures in clinical cases involving nitroimidazoles have been increasingly reported. Consequently, identifying therapeutic alternatives is necessary. Medicinal plants have traditionally been used as antiparasitic compounds, but systematic evaluation under controlled experimental conditions is often lacking. Here, we evaluated the *in vitro* efficacy of *Tabebuia avellanedae* dry and hydroalcoholic extracts, as well as one of its active compounds, β-lapachone, as potential treatment against *G. duodenalis* infection. We observed effective antigiardial activity for all tested compounds, with β-lapachone exhibiting lower IC_{50} values than

metronidazole. Cytotoxic effects often limit therapeutic concentration windows of opportunity, and choosing an informative model to assess them is not straightforward. In the present case, only *T. avellanedae* hydroalcoholic extract showed no cytotoxicity on tumoral human intestinal Caco-2 cell line, and only a trend of inhibition when tested on canine epithelial kidney MDCK cells. To introduce a more physiological test system, we used *in vitro G. duodenalis* infection experiments in a trans-well set-up using organoid derived monolayers (ODM) to assess at the same time drug efficacy against the parasite and safety on primary human intestinal epithelia, a likely surrogate for *in vivo* conditions. Our studies using this model point towards the potential therapeutic opportunity for non-systemic applications of *T. avellanedae* extracts and a relevant ingredient of these, β-lapachone. The data suggest that ODM co-cultures with *G. duodenalis* are suitable for testing antigiardial compounds, providing a more informative *in vitro* model before progressing to *in vivo* tests.

Key words: *Giardia duodenalis, Tabebuia avellanedae, in vitro* activity, safety

1. Introduction

Giardia duodenalis (syn. *G. intestinalis* or *G. lamblia*) is a widespread protozoan parasite able to infect the proximal small intestine of more than 40 mammals, including humans [1]. Infection occurs via the faecal-oral route through the accidental ingestion of *G. duodenalis* cysts, the environmentally resistant stage of the parasite, either by direct contact with infected faeces or by ingestion of contaminated water or food (such as fresh produce). The waterborne route is most often associated with human infection. *G. duodenalis* can be classified in eight groups, or Assemblages, based on the genetic profiles and host specificity. Assemblages A and B display a certain level of zoonotic potential, as they can be isolated from both humans and animals (especially sub-assemblage AI and B), whereas Assemblages C and D are only found in canids, E in ungulates, F in felids, G in rodents and H in pinnipeds [2]. In humans, infection can reach prevalence rates of 2-7% in high income countries and 20-60% in low-income countries and is generally high in settings with poor hygiene [3,4]. Infection can range from asymptomatic cases to chronic diarrhoea,

malabsorption, and weight loss [5]. Moreover, *G. duodenalis* interferes with the body's ability to absorb fat, lactose, vitamin A, and vitamin B12, causing progressive weight loss, malnutrition conditions and cognitive development problems, especially in children [6,7]. In humans no effective vaccine is available; approved drugs include six classes of compounds: 5-nitroimidazoles, benzimidazoles derivatives, quinacrine, furazolidone, paromomycin and nitazoxanide [1]. The 5-nitroimidazole metronidazole (MTZ) is the first-choice treatment, with recovery rates exceeding 90% [8,9]. Despite its efficacy, this molecule may exhibit adverse side effects (e.g., headache, nausea, glossitis, neutropenia) and has carcinogenic, teratogenic, and embryogenic properties [8,10]. In recent years, treatment failures with MTZ have been reported in 10-20% of patients [11,12] and an increasing number of cases of giardiasis refractory to treatment, mainly with 5-nitroimidazoles, have been observed in humans. This suggests that mechanisms of tolerance to all major antigiardial drugs may not be uncommon in the parasite [12–14]. Therefore, new therapeutic alternatives for giardiasis are currently being investigated. For instance, new potential compounds are being identified based on ethnopharmacological concepts regarding the efficacy of medicinal plants and/or their active molecules or structurally similar analogues [15–17]. *Tabebuia avellanedae* (Lorentz ex Griseb.) (syn. *Tabebuia impetiginosa* or *Handroanthus impetiginosus* or *Red Lapacho*) (Fam. Bignoniaceae) is a tree native to Central and South America, widely used in local and traditional phytomedicine to treat bacterial, fungal, and protozoan infections [18]. Traditionally, *T. avellanedae* is consumed as tea, made from the inner bark of the tree. The traditional preparation involves making a decoction from half to one cup of bark and/or wood, taken orally two to four times a day [19]. Further, *T. avellanedae* applications include douching to treat yeast infections; topical application to treat fungal skin infections; use of tincture, ingested orally or applied to mucous membranes to reduce inflammation [20]. In addition, the main bioactive components of *T. avellanedae*, naphthoquinones (e.g., lapachol, β-lapachone, and α-lapachone), have shown cytotoxicity against several tumor cell lines *in vitro* [21–23] and *in vivo* [24,25], as well as antibacterial activity (e.g., against *Clostridium perfringens, Escherichia coli,* and *Helicobacter pylori*) [26,27] and anti-protozoan activity against *Trypanosoma cruzi*, *Leishmania* spp., and *Plasmodium falciparum,* in both *in vivo* and *in vitro*

studies [28–32]. Noteworthy, previous research showed that β-lapachone induces *in vitro* cell death and morphological abnormalities in *G. duodenalis* and stimulate the expression of encystation markers [33].
Here, we evaluated the antigiardial efficacy of *T. avellanedae* bark extracts and its bioactive compound β-lapachone. We also tested their cytotoxicity on common cell lines and characterized the metabolite profile of *T. avellanedae* extracts. Furthermore, the effect of these compounds was compared under more physiological conditions using a well-established stem cell-derived intestinal organoid model on trans-well filter as a surrogate infection model.

2. Materials and methods

2.1 Natural and chemical compounds

Tabebuia avellanedae dry extract (TD) and hydroalcoholic extract (EtOH 45%) (TH) were provided by Deakos SRL (La Spezia, IT). β-lapachone (2,2-Dimethyl-3,4-dihydro-2H-benzo[h]chromene-5,6-dione, $C_{15}H_{14}O_3$) (L2037) and metronidazole (MTZ) (M3761) were purchased from Merck (Sigma-Aldrich, Milan, IT). For stock solutions, β-lapachone, MTZ and TD were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 10 mM for β-lapachone and MTZ, and 200 mg/ml for TD; *Tabebuia avellanedae* hydroalcoholic extract was stocked undiluted at 100 mg/ml, according to the manufacturer's datasheet. All compounds were stored in dark and frozen condition, except for TH, which was stored at room temperature according to the manufacturer's instructions.

2.2 Parasite isolates and cultivation

The *G. duodenalis* isolates used in this study are listed in Table 1. Trophozoites were axenically grown at 37° C in flat-sided 10 mL screw-cap tubes (Nunclon; Thermo Fischer Scientific, Waltham, MA, USA) filled with at least 10 mL filter-sterilized Keister's modified TYI-S-33 medium, supplemented with 10% adult bovine serum (Gibco, Grand Island, NY), 100 µg/mL streptomycin and 100 U/mL penicillin (Capricorn, Frederick, MD) and 0.05% bovine bile (Sigma-Aldrich, St. Louis, MO)[34]. Confluent cultures were sub-cultured two to three times a week by placing tubes on ice for 20 minutes to detach trophozoites, then transferring 1:10-1:100 of the culture to fresh medium.

Isolate	Assemblage	Laboratory	Reference
WBC6 (ATCC-50803)	AI	IT, DE	
GS/M (ATCC-50581)	B	IT, DE	
P424/A5	B	DE	[35]

Table 1. List of *Giardia duodenalis* isolates used in the present work. IT=Italy, DE=Germany

2.3 Mammalian cells lines and culture

Human colon adenocarcinoma cell lines (Caco-2) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% inactivated foetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin and 1% non-essential amino acids solution [36,37]. Madin-Darby canine kidney cells (MDCK) were cultured in the same medium conditions but without the addition of non-essential amino acids solution [38]. Cells were cultivated at 37° C in a humidified 5% CO_2 atmosphere until reaching approximately 80% confluence before passage. Cell culture media and supplements were all purchased from Euroclone (Milan, IT).

2.4 Intestinal Organoid Derived Monolayers (ODM)

Human duodenal Organoid-Derived Monolayers (ODM) were prepared from an established and well-characterized organoid culture, as previously described [39–42] Briefly, ODM were generated on Cultrex-coated (Bio-Techne, Minneapolis, MN, USA) trans-well cell culture inserts (0.6 cm^2, 0.4 μm pores; Merck-Millipore, Burlington, MA, USA). To achieve this, 3D stem cell-enriched intestinal organoid cultures (previously obtained from small intestine biopsy specimen from a healthy volunteer at Charité Universitätsmedizin Berlin, with ethics approval #EA4-015-13 by German authorities, for details see [40])were collected in Advanced DMEM/F-12, centrifuged, and mechanically disrupted to generate a single-cell suspension, following exactly the previously established procedure [39]. The resulting cells were added to the apical compartment of the insert ($5x10^5$ cells/filter). To obtain ODM with a physiological cell composition, consisting mainly of enterocytes [40], differentiation was induced using ODM differentiation medium. This medium consisted of 20% (v/v) R-Spondin 1-conditioned medium, 10% (v/v) Noggin-conditioned medium, 50 ng/mL

Human Epidermal Growth Factor (hEGF), 1 mM HEPES, 2 mM GlutaMax, 1× P/S (100 U/mL penicillin and 100 µg/mL streptomycin), 1× N2, 1× B27, 1 mM *N*-acetyl-L-cysteine, and 10 mM nicotinamide in Advanced DMEM/F-12 [39,40]. Both compartments of the trans-well system received the ODM differentiation medium, which was refreshed every 2 to 3 days [40]. Transepithelial electric resistance (TEER) measurements were made on a 37°C heating block using a Millicell ERS-2 Voltohmmeter (Merck-Millipore) that was equipped with an Ag/AgCl electrode (STX01; Merck-Millipore) [39,40]. After 8 days, the ODM reached a confluent stage, as confirmed by TEER, and were used for subsequent applications.

2.5 In vitro cytotoxicity assays

The drug susceptibility of *G. duodenalis* trophozoites was determined using a previously described bioluminescent ATP content assay [43] with some modifications. The assays were carried out in 96-well microplates with flat clear bottoms. Trophozoites from a log-phase culture were harvested by chilling on ice and counted in a hemocytometer (Kova™, Thermo Fischer Scientific, Waltham, MA, USA). Trophozoites were seeded at $0.5x10^5$/well in 200 µl of modified TYI-S-33 medium. Two-fold serial dilutions of the compounds were prepared in DMSO or EtOH 45% at 100X concentration and individually added to each well. All wells contained DMSO 1% and EtOH 0.45%. Cells were incubated at 37°C for 48 hours under anaerobic conditions by placing the microplates in Aerogen Oxoid jars containing appropriate reaction bags (Oxoid #AN0025). After 48 hours, trophozoites viability was determined by the bioluminescent ATP content assay, according to the manufacturer's instructions (CellTiterGlo 2.0, Promega Italia, Milan, IT). Each experiment was done in triplicate, and at least three biological replicates were performed.

To evaluate drug cytotoxicity against mammalian cells, Caco-2 and MDCK cells were seeded onto 96-well microplates in 100 µL of culture medium at a density of $1x10^4$cell/well and $1.5x10^4$ cell/well, respectively. Both cell lines were allowed to attach to the plate for 24 hours. After 24 hours, the medium was replaced with fresh medium containing the same compounds at the same concentrations used for testing *G. duodenalis* trophozoites. Each concentration was tested in triplicate, and viability measured at 6, 12, 24 or 48 hours. At the end of incubation time, 10 µL/well of Cell Counting

Kit-8 (CCK8) solution (Target Molecule Corp, Boston, USA) was added and incubated at 37 °C for 2 hours for Caco-2 and 1 hour for MDCK until the colour turned orange. CCK-8 is an assay for assessing cell viability and cytotoxicity; it utilises WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], a water-soluble tetrazolium salt reduced by cellular dehydrogenases to a coloured formazan dye. The amount of formazan generated is directly proportional to the number of living cells. The absorbance was measured using a plate spectrophotometer (Infinite 200 Pro-Tecan, Mennedorf, CH) at 450 nm. All 96-well microplates included media-only, and vehicle (DMSO and EtOH) control. Each experiment was done in triplicate, and at least three biological replicates were performed.

2.6 UHPLC-IMS-HRMS analysis of TH

To investigate the metabolite profile of TH, an untargeted screening was performed. For this purpose, a diluted aliquot of TH (1:100) in a 70% ethanol solution was analysed on an ACQUITY UHPLC I-Class system (Waters Corporation) coupled to a Synapt XS HDMS mass spectrometer (Waters Corporation) using an electrospray ionization interface operating in positive mode (ESI+). Data acquisition and processing were performed using UNIFI software (Version 3.1.0.16, Waters Corporation). Chromatography separation was performed using a BEH C18 column (2.1 x 100 mm, 1.7 µm particle size; Waters Corporation), mobile phases A = Water (LC-MS grade purchased from Sigma-Aldrich) and B = Acetonitrile (LC-MS grade purchased from Sigma-Aldrich), both containing 0.1% of formic acid (purchased from Sigma-Aldrich). The gradient started with 20% acetonitrile and reached 100% in 10 minutes; then, the system returned to initial conditions in 1 minute and finally equilibrated for 2 minutes, resulting in a total runtime of 13 minutes. A flow rate of 0.4 mL/min, a column oven temperature of 40 °C, and a sample injection volume of 2 µL was selected. A vial containing 100 µL of the 70% ethanol solution was analysed under the same conditions as the TH aliquot and was used as a procedural blank to remove solvents and system interferences.
After chromatographic separation, the metabolites were detected by the combination of ion mobility separation coupled to high-resolution mass spectrometry (IMS-HRSM). Regarding the IMS-HRMS setup, the

capillary voltage was set at 2 kV, the source temperature was set to 120 °C, and the desolvation gas temperature was set to 20 °C with a flow rate of 300 L/h. The mass spectrometer was operated in ion mobility mode ($HDMS^E$) for acquisition. $HDMS^E$ experiments provide the acquisition of a low-energy function (LE) and high energy function (HE); 6 eV was set as collision energy for the LE function, and the collision energy ramp from 25 to 50 was set for the HE function. $HDMS^E$ is a data-independent acquisition method that can determine the drift time (DT) of each ion. DT can be converted into a collision cross-section value (CCS, $Å^2$), which provides information about the structure of a chemical compound and its three-dimensional conformation [44]. The CCS value represents an additional level of identification for unknown compounds. The combination of ion mobility experiments with accurate mass determination and mass spectra allows for the elucidation of unknown substances in real samples with a high level of confidence. Calibrations of mass axis and CCS were performed monthly using a "Major Mix Calibration Sample Kit" supplied by the vendor (Waters Corporation). A Leucine-Enkephalin solution (100 ppb, purchased from Waters Corporation) in ACN:H_2O (50:50, *v/v*) with 0.01% of formic acid was used as lock mass to ensure the robust accurate mass measurement throughout runs.

The Synapt instrument data was imported to UNIFI software. The following key parameters were set on the UNIFI platform for data processing: mass error 10 ppm, fragment match tolerance 2 mDa, look for in-source fragments, maximum candidates per sample to keep: 10000, adducts to search: H^+. The "Binary Sample Compare" tool was applied on UNIFI between the TH and procedural blank spectra. This approach allowed for the identification of unique metabolites present in TH by subtracting the compounds present in the procedural blank from those present in TH. For the elucidation of TH metabolites, the experimental results were compared with the theoretical information available in the "Natural Products Profiling CCS Library", an online library integrated into UNIFI software that contains compounds belonging to the following compound classes: alkaloids, coumarins, flavones, isoflavones, macrolides, peptides, synthetic derivatives, and terpenoids. Only substances whose m/z and CCS values, as well as mass spectra, molecular formulas, and structures matched those contained in the library were selected as possible

metabolites.

2.7 Cytotoxicity evaluation on non-infected ODM and ODM infected with G. duodenalis trophozoites

For cytotoxicity assays, the compounds were added to the apical compartment medium of 8-day-old ODM at final concentration of 2, 1, and 0.25 mg/ml for TD; 1, 0.5, and 0.25 mg/ml for TH; and 100, 25, and 2.5 μM for β-lapachone. As a positive control for cell death, 2 μM staurosporine (Tocris, Bristol, UK), a non-selective apoptosis inducer, was used in all experiments [40]. After 48 hours, cell viability was evaluated using a luminescent assay that assesses the quantity of ATP present in the culture as an indicator of metabolically active cells (CellTiterGlo 2.0, Promega, DE). Furthermore, to test the effects of TH and β-lapachone on ODM infected with *G. duodenalis*, the apical compartment medium of 8-day-old ODM was replaced with TYI-S-33 the evening before infection to adapt the cells to the *G. duodenalis* medium. TYI-S-33 was renewed prior to infection (i.e., TYI-S-33 in the apical compartment and organoid differentiation medium in the basal compartment), and $2x10^5$ trophozoites (WBC6 strain) were added apically to obtain an approximate multiplicity of infection (MOI) of 1 [40]. After that, the compounds were added to the apical compartment medium at concentrations of 1, 0.5, and 0.25 mg/ml for TH, and 100, 25, and 2.5 μM for β-lapachone. After 48 hours, trophozoites were harvested by incubating the plates on ice for at least 20 minutes, followed by counting using a Neubauer cell counting chamber (Kova™, Thermo Fisher Scientific, Waltham, MA, USA). For both non-infected and infected ODM experiments, TEER measurements were made on the day of the treatment (day 0) and 48 hours after the treatment (day 2). At the end of the experiment, selected filters were fixed with 4% paraformaldehyde for 15 min, washed in PBS, and used for immunofluorescence analysis (IFA) to confirm the integrity of the cell monolayer. For IFA, cells were permeabilized with 0.025% TritonX-100 in PBS for 30 minutes, followed by blocking with 1% BSA in PBS for 1 hour and subsequent staining using anti-ZO1 antibody (1:250 dilution, #610967, BD Biosciences) in blocking buffer overnight at 4°C. For detection and counterstaining, the cells were then incubated for 1 hour with a secondary antibody solution (1:500 Goat anti-mouse Alexa 647, #A28181,

ThermoFisher) containing Phalloidin i488 (1:1000, ab176753, Abcam) and DAPI (1:1000 from 10 mM stock). Stacked images were taken on a Leica Mica system, and images were analysed using LasX software (Leica). The experiments were done in triplicates and repeated at least two times.

2.8 Statistical analysis

The half maximal inhibitory concentrations (IC_{50}) for *G. duodenalis* trophozoites and the 50% cytotoxic concentrations (CC_{50}) were determined using non-linear regression analysis; the selectivity index (SI) was calculated as the ratio of IC_{50} to CC_{50} for each *G. duodenalis* isolate in relation to both MDCK and Caco-2 cell lines. Drug cytotoxicity against mammalian cells was assessed by calculating the percentage of viable cells, normalizing the absorbance of treated wells to the vehicle control set at 100% viability. Results were presented as mean ± standard deviation (SD) and analysed using one or two-way ANOVA with Tukey's multiple comparison test. A p-value of less than 0.05 was considered statistically significant. All the analyses were performed using GraphPad Prism® version 9 (GraphPad Software, San Diego, USA).

3. Results

3.1 Activity of T. avellanedae extracts and β-lapachone against G. duodenalis trophozoites, Caco-2 and MDCK cell lines

T. avellanedae dry (TD) and hydroalcoholic (TH) extracts both displayed effective activity against *G. duodenalis* trophozoites, with comparable 48-hour IC_{50} values irrespective of the isolate or the Assemblage tested (Table 2). Additionally, β-lapachone exhibited potent antigiardial activity, in agreement with a previous report [33]. Both β-lapachone and MTZ showed a broader range of IC_{50} values across the isolates tested, particularly concerning the laboratories where they were maintained (Table 2). Noteworthy, β-lapachone proved to be more potent than MTZ, consistently displaying slightly lower 48-hour IC_{50} values per isolate, with tight confidence intervals for all measurements suggesting high data reliability.

	WBC6*	**WBC6°**	**GS/M***	**GS/M°**	**P424/A5°**
TD (mg/ml)					
IC_{50} (95% CI)	1.50 (1.45 - 1.55)	1.32 (1.21 - 1.43)	1.28 (1.24 - 1.33)	1.16 (1.12 - 1.19)	1.08 (1.06 - 1.10)
TH (mg/ml)					
IC_{50} (95% CI)	1.38 (1.34 - 1.42)	1.27 (1.15 - 1.40)	1.19 (1.16 - 1.22)	1.19 (1.14 - 1.24)	1.14 (1.11 - 1.17)
β-lapachone (µM)					
IC_{50} (95% CI)	6.2 (4.41 - 7.04)	2.43 (2.18 - 2.71)	5.90 (5.26 - 6.71)	3.97 (3.51 - 4.42)	3.16 (2.81 - 3.56)
MTZ (µM)					
IC_{50} (95% CI)	6.77 (6.11 - 7.50)	4.67 (4.07 - 5.35)	7.77 (7.23 - 8.36)	6.55 (5.36 - 8.00)	5.13 (4.36 - 6.03)

Table 2. Activity of *Tabebuia avellanedae* dry extract (TD), *Tabebuia avellanedae* hydroalcoholic extract (TH), β-lapachone and metronidazole (MTZ) against *Giardia duodenalis* trophozoites, represented as inhibitory concentration 50 (IC_{50}) with a 95% Confidence Interval (CI) for each compound. IC_{50} values are expressed in mg/ml for TD and TH and in µM for β-lapachone and MTZ. *=*G. duodenalis* isolates maintained in the IT laboratory; °= *G. duodenalis* isolates maintained in the DE laboratory.

The compounds were then tested against mammalian epithelial cell lines of human and canine origin to evaluate their potential cytotoxicity. Caco-2 and MDCK cell lines were chosen as both have been widely used to assess the permeability of compounds in *in vitro* conditions for pharmaceutical use [45,46], and because Caco-2 cells are commonly used for *in vitro* interaction studies with *G. duodenalis* trophozoites [47]. TD showed cytotoxic effects for both cell lines at concentration higher than 1 and 2 mg/ml, respectively, with a limited effect with respect to the incubation time (Supplementary Figure S1 and S2). The 48-hour CC_{50} for both cell lines (Supplementary Figure S3) was in the same range as 48-hour IC_{50} measured for *G. duodenalis* trophozoites (Table 2 and 3).

	Caco-2	MDCK
TD (mg/ml)		
CC_{50} (95% CI)	1.71 (1.51 - 1.92)	1.73(1.56 - 1.93)
TH (mg/ml)		
CC_{50} (95% CI)	>100	>100
β-lapachone (μM)		
CC_{50} (95% CI)	4.19 (3.41 - 5.14)	4.11 (3.69 - 4.58)

Table 3. Activity of *Tabebuia avellanedae* dry extract (TD), *Tabebuia avellanedae* hydroalcoholic extract (TH), and β-lapachone against Caco-2 and MDCK cell lines after 48 hours represented as cytotoxicity concentration 50 (CC_{50}) with a 95% Confidence Interval (CI) for each compound. CC_{50} values are expressed in mg/ml for TD and TH and in μM for β-lapachone.

β-lapachone also exhibited strong cytotoxic effects in both immortalised cell lines within the low μM range (Supplementary Figure S1 and S2). Similar to TD, the 48-hour CC_{50} for β-lapachone was of the same order of magnitude as the 48-hour IC_{50} for *G. duodenalis.* Based on these results, a mean SI <1.7 was obtained for TD and β-lapachone for either of the cell lines (Table 4). Thus, under these assumptions, the compounds would be judged as unsuitable as antigiardial agents. In contrast, TH did not demonstrate significant cytotoxicity in either cell line, with SI for TH >100 since no CC_{50} could be calculated (Table 4). Although a trend toward reduced cell viability was noted in MDCK cells at the highest concentration, however, this was not statistically significant (Supplementary Figure S1 and S2).

	WBC6*	WBC6°	GS/M*	GS/M°	P424/A5°
TD					
MDCK	1.16	1.32	1.35	1.50	1.61
Caco-2	1.14	1.30	1.33	1.47	1.58
TH					
MDCK	>100	>100	>100	>100	>100
Caco-2	>100	>100	>100	>100	>100
β-lapachone					
MDCK	0.66	1.69	0.70	1.04	1.30
Caco-2	0.68	1.72	0.71	1.06	1.32

Table 4. Selectivity index (SI) of each compound for each *G. duodenalis* isolate in relation to MDCK and Caco-2 cell lines. *=*G. duodenalis* isolates maintained in the IT laboratory; °= *G. duodenalis* isolates maintained in the DE laboratory.

3.2 UHPLC-IMS-HRMS analysis of TH identifies potential antigiardial molecules

Following the promising results obtained with TH, the profile of metabolites present in the extract was screened by untargeted metabolomic fingerprinting approach using the combination of Ultra-Performance Liquid Chromatography-Ion Mobility Separation coupled to High Resolution Mass Spectrometry (UHPLC-IMS-HRMS*)*. Supplementary Figure S4 shows the Base Peak Intensity (BPI) chromatogram of the TH. After applying the "Binary Sample Compare" tool, a list of 45 candidate mass metabolites was found in TH by the UNIFI software. After querying the "Natural Products Profiling CCS" online library, a shortlist of 22 metabolites was identified with high confidence through the combination of ion mobility experiments, accurate mass determination, and mass spectra, even without the comparison of retention times, fragmentations, and CCS values with commercially available standards (Table 5). Noteworthy, β-lapachone was among the identified metabolites, but the low toxicity of TH in mammalian cells compared to purified β-lapachone might suggest that the presence of other metabolites either partially counteract the cytotoxic effect of β-lapachone in mammalian cell or indicates that β-lapachone is present at sub-toxic concentration for mammalian cells, with its efficacy against *G. duodenalis* improved by other metabolite.

No.	Component name	Theoretical neutral mass (Da)	Experimental neutral Mass (Da)	Mass error (mDa)	m/z	RT (min)	Theoretical fragments found	Adducts	Theoretical CCS (Å²)	Experimental CCS (Å²)	CCS Delta Error (%)
1	(-)-Ouabain	584.2833	584.285	1.3	585.292	6.84	2	+H	236.48	237.76	0.54
2	(±)-Abscisic acid	264.1362	264.136	0.2	265.144	3.81	1	+H	156.12	158.74	1.68
3	Auraptene	298.1569	298.154	-3	299.161	11.74	1	+H	168	169.44	0.86
4	Austricin	262.1205	262.12	-1	263.127	4.68	2	+H	156.72	156.33	-0.25
5	Carnitine	161.1052	161.105	0	162.112	0.57	2	+H	132.59	134.29	1.28
6	Dehydrocostus lactone	230.1307	230.131	-0.2	231.138	5.26	2	+H	150.67	153.04	1.57
7	Eriodictyol	288.0634	288.064	0.6	289.071	2.82	1	+H	164.33	167.31	1.81
8	Flavokawain A	314.1154	314.114	-1.9	315.121	2.49	4	+H	172.7	173.93	0.71
9	Flavokawain B	284.1049	284.106	0.7	285.113	2.64	1	+H	163.94	164.39	0.28
10	Hydrocotarnine	221.1052	221.105	0	222.113	0.6	4	+H	146.99	147.41	0.29
11	Indirubin	262.0742	262.077	2.3	263.084	4.9	1	+H	151.7	152.16	0.3
12	Isorhamnetine-3-glucoside	478.1111	478.115	3.8	479.122	2.5	1	+H	207.49	207.34	-0.07
13	Isorhamnetine-3-rutinoside	624.169	624.173	4	625.18	3.84	1	+H	234.77	237.76	1.27

14	Luteolin	286.0477	286.048	0.1	287.055	2.19	1	+H	159.75	158.68	-0.67
15	Mycophenolic acid	320.126	320.124	-2.5	321.131	2.51	2	+H	166.33	163.26	-1.84
16	Phloretin	274.0841	274.084	-0.6	275.091	5	1	+H	159.76	158.98	-0.49
17	Robinetine	302.0427	302.041	-1.8	303.048	0.61	1	+H	164.51	163.12	-0.85
18	Salsoline	193.1103	193.111	0.3	194.118	0.6	1	+H	144.86	143.08	-1.23
19	Scopoletin	192.0423	192.042	-0.4	193.049	2.48	1	+H	133.33	134.26	0.7
20	Sinensetine	372.1209	372.118	-2.6	373.126	2.47	1	+H	187.17	189.47	1.23
21	Stachydrine	143.0946	143.095	0.4	144.102	0.58	1	+H	127.83	125.69	-1.67
22	β-Lapachone	242.0943	242.095	0.3	243.102	11.72	1	+H	150	148.36	-1.09

Table 5. List of metabolites identified through untargeted screening of *Tabebuia avellanedae* hydroalcoholic extract (TH).

3.3 Activity of T. avellanedae extracts and β-lapachone on non-infected ODM and ODM infected with G. duodenalis trophozoites

As organoid derived primary cells represent a more physiological model than immortalised cell lines, we used this model for further evaluating the cytotoxicity of the compounds and to determine their anti-*G. duodenalis* efficacy under co-culture conditions with host cells. Our well-characterised ODM model is based on a trans-well filter that generates strongly polarised and very tight epithelial layers akin to intestinal epithelia [40–42]. In the framework of this study the model has three major advantages: i) β-lapachone is a known anti-cancer drug, and thus its effects on cancer-derived cell lines like Caco-2 cannot be correlated with potential effects on primary cells; ii) ODM consist of differentiated primary intestinal epithelial cells that are resistant to TYI-S-33, making them suitable for conducting infection experiments under optimal conditions for *G. duodenalis* without harming the host cells [40]; iii) the cells in the ODM system are strictly polarised with proper junction complexes, thus more accurately mimicking the epithelial barrier [41].

Compounds at any of the three chosen concentrations did not affect the metabolic activity of ODM cells, suggesting, in contrast to the immortalized cell lines, no cytotoxicity towards the ODM in comparison to the vehicle control (Figure 1). Furthermore, the monolayer barrier integrity was monitored using TEER measurements, which showed no impairment upon treatment with the tested compound concentrations (Figure 1).

Since TH proved to be a safer and more standardised formulation compared to TD, we next tested the effect of β-lapachone and TH on ODM infected with *G. duodenalis* trophozoites to determine whether the drug response would be comparable to the result without host cells. We used a trophozoite concentration known not to affect host cell barrier integrity in the tested time frame [40]. The results indicate that both β-lapachone and TH (Figure 2) effectively reduce the number of trophozoites as their concentrations increase, without affecting host cell barrier integrity as measured by TEER and immunofluorescence microscopy after 48 hours of treatment (Figure 2 and Figure 3).

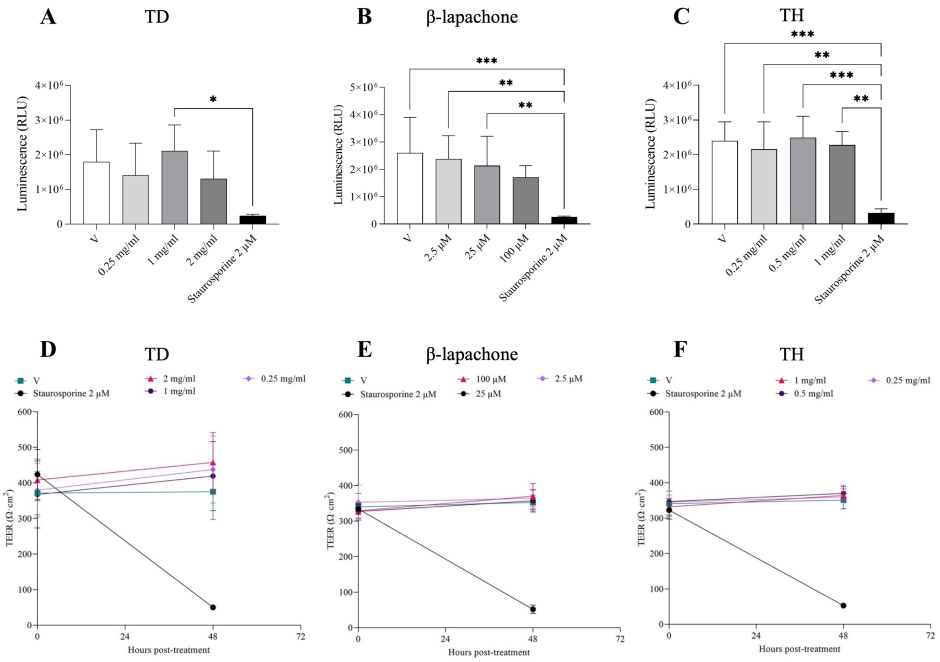

Figure 1. Effects of different concentrations of *Tabebuia avellanedae* dry extract (TD), hydroalcoholic extract (TH) and β-lapachone on ODM. A-C) ODM viability. Staurosporine at 2 µM, a nonselective apoptosis inducer, was used as positive control in all respective experiments [40], along with vehicle (DMSO and EtOH) control (V). After 48 hours, cell viability was assessed using a luminescent assay to measure ATP levels in the culture as an indicator of metabolically active cells. Each experiment was done in duplicate and repeated at least twice. Results are expressed as mean ± SD and were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was set at $p<0.05$; ***$p<0.001$, **$p<0.01$, *$p<0.05$ vs vehicle control (V), as indicated in figure. D-F) Transepithelial electric resistance (TEER) of ODM following 48 hours of treatment with vehicle (V) or the indicated compounds: *T. avellanedae* dry extract (TD), *T. avellanedae* hydroalcoholic extract (TH) and β-lapachone. Blank electric resistance (cell-free trans-well insert) was subtracted from raw resistance values and standardised for 1 cm^2 surface area.

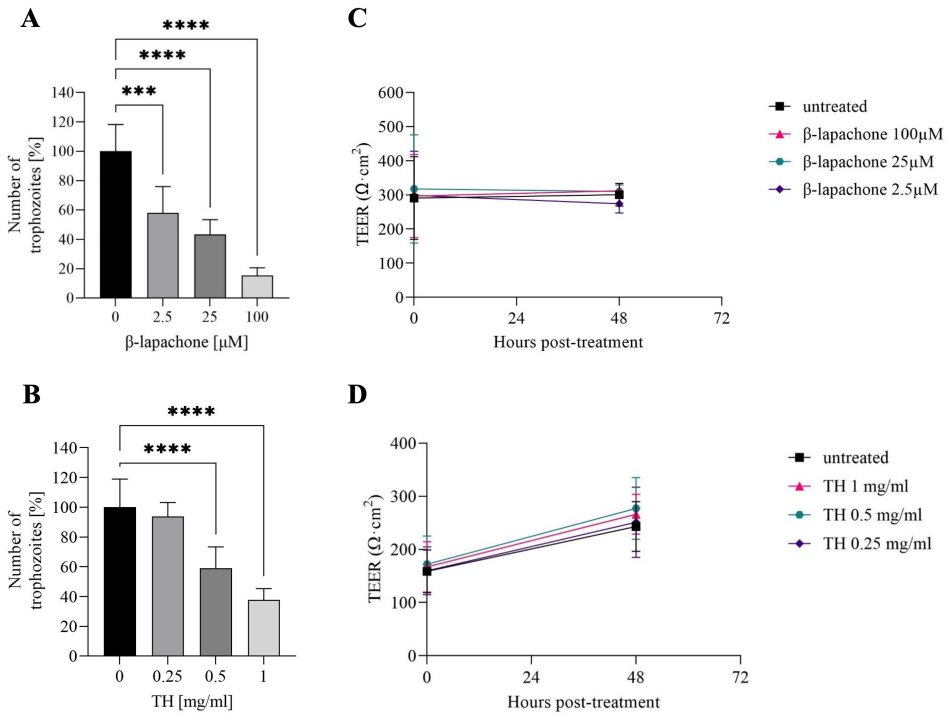

Figure 2. Evaluation of β-lapachone and *Tabebuia avellanedae* hydroalcoholic extract (TH) on *Giardia duodenalis* WBC6 trophozoites cultured on ODM. A-B) Parasite count after 48 hours of culture with or without the indicated compounds at different concentrations. Statistical significance was set at $p<0.05$; **** $p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$ vs 0 μM or 0 mg/ml, as indicated in figure. C-D) Transepithelial electric resistance (TEER) evaluation in ODM after 48 hours of culture with *G. duodenalis* trophozoites, with or without treatment using the indicated compounds at different concentrations. Blank electric resistance (cell-free trans-well insert) was subtracted from raw resistance values and standardised for 1 cm^2 surface area. Representative experiments conducted in triplicates are shown. Each experiment was repeated at least twice with similar results.

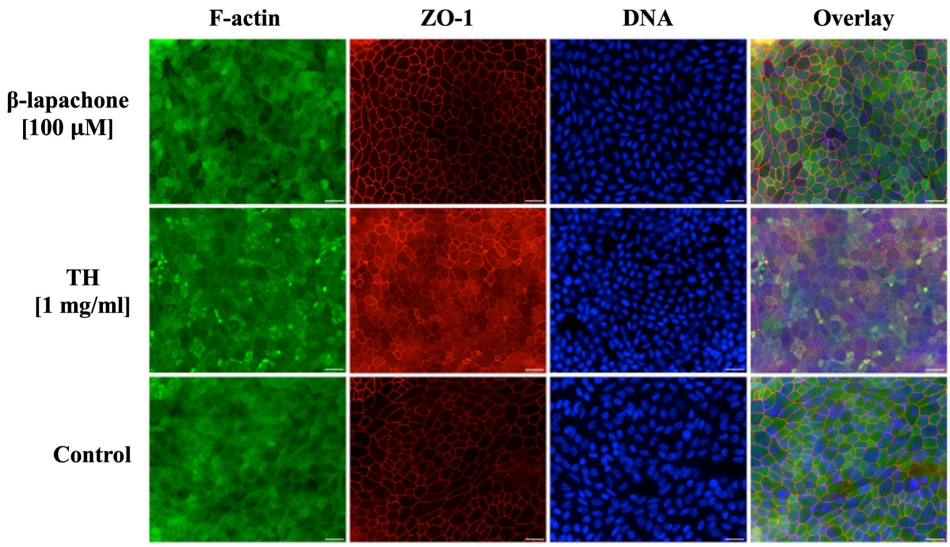

Figure 3. Immunofluorescence analysis of ODM 48 hours post *G. duodenalis* infection and various treatment regimens shows similarly intact barrier integrity. ODM were infected with $2x10^5$ trophozoites (WBC6) and treated with β-lapachone or *T. avellanedae* hydroalcoholic extract (TH) as indicated, or non-treated (control) for 48 hours. After detachment of the parasites, the filters were fixed and stained with Phalloidin (F-actin, green), anti-ZO-1 antibody (ZO-1, red) and DAPI (DNA, blue). Images were captured on a Leica Mica imaging system with optimised optical stacks and processed using Leica Las X software. Note that, due to the polarised nature of the ODM, nuclei are presented from separate Z-layers. Further, the filters are derived from different experiments and staining procedures, resulting in varying image quality. However, the images highlight comparable and intact barrier integrity across all samples.

3. Discussion

Despite the global prevalence and impact of *G. duodenalis* on public health, effective drug treatments for giardiasis are limited and associated with treatment failures. Here, we provide evidence that compounds from the known medicinal plant *T. avellanedae* possess effective antigiardial activity *in vitro*, which may offer potential new treatment options. Both plant extracts and the active compound β-lapachone, also present in the hydroalcoholic extract of *T. avellanedae*, as confirmed by our metabolites analysis, were effective against isolates from the two zoonotic *G. duodenalis* Assemblages AI (WBC6) and B (GS/M and P424/A5). *T. avellanedae* hydroalcoholic extract and β-lapachone proved to be safe when tested on ODM, which mimics physiological conditions more closely

than commonly used epithelial cell lines (i.e., Caco-2 and MDCK). Moreover, for the first time, the efficacy of new potentially antigiardial compounds was confirmed in a *Giardia*-organoid co-culture system.

Concerning the efficacy of the different compounds on *G. duodenalis*, the IC_{50} values measured for TD and TH are in mg/ml range, which appears to be a relatively high concentration when compared to other reports on natural hydroalcoholic extracts tested against *G. duodenalis* [48,49]. However, the limited number of studies on the efficacy of natural products against *G. duodenalis* do not allow any strong conclusions. Most of the research has focused on essential oils or aqueous extracts, with compounds primarily tested on cysts rather than trophozoites [50,51]. The relatively similar IC_{50} values of both extracts on *G. duodenalis* trophozoites suggest no differences in solubility of active metabolite(s) in the two solvents used (DMSO or ethanol), although β-lapachone has a higher solubility in alcoholic solvents compared to water-based solvents [52]. This also suggests that a rather complex mixture of active metabolites in both extracts could mediate the reported antigiardial effect in addition to β-lapachone. Our metabolite analysis of TH supports, with high confidence, the occurrence of 22 metabolites, confirming the presence of β-lapachone as well as luteolin, a flavonoid found in another extract tested against *G. duodenalis* that has shown effectiveness [53]. Lutein's mechanism of action appears to cause damage to the cytoskeleton due to alterations in the expression and distribution of α-tubulin, particularly in the ventral disk, which is a key structure for adhesion and pathogenesis [53]. Corrêa *et al*. [33] found that treatment with β-lapachone in *G. duodenalis* induces several apoptotic morphological changes, such as cell shrinkage, chromatin condensation, membrane blebbing, and vacuolization. Although the parasite is amitochondrial, β-lapachone exhibits characteristics of both apoptotic and autophagic cell death, suggesting a complex mechanism of action in *Giardia* [33]. In cancer cells, β-lapachone has been shown to alter the cell redox state by undergoing a futile NAD(P)H:quinone oxidoreductase 1 (NQO1)-mediated redox cycle, which results in high levels of superoxide and subsequent peroxide formation that eventually kill the cell [54]. A similar mechanism of action for β-lapachone can also be hypothesized for G. *duodenalis*. Indeed, another naphthoquinone, 2-methy-1,4-naphthoquinone (menadione) is highly toxic for *G. duodenalis* under microaerophilic

condition by redox cycling and ROS generation [55]. Although the antigiardial activity of other redox cycler compounds, such as NBDHEX, has been also demonstrated [56], the exact mechanism of action of β-lapachone has yet to be proven. Furthermore, among the 22 identified metabolites, some have been effectively tested against other parasites, such as auraptene against *Haemonchus contortus* [57], eriodictyol (specifically 6,8-diprenyleriodictyol) against intracellular amastigotes of *Leishmania amazonensis* [58], flavokawain B against *Trypanosoma cruzi* and *Trypanosoma brucei*, and non-toxic to Hep G2 cells [59], as well as indirubin [60]. It would be interesting to test the efficacy of these molecules also against *G. duodenalis.*

For the first time, the efficacy of β-lapachone was compared to the reference drug MTZ, the first choice for the treatment of giardiasis [8,9], showing a slightly lower IC_{50} value for β-lapachone than for MTZ in all *G. duodenalis* isolates tested. We tested various isolates, as previous studies have noted significant variability in drug susceptibility between assemblages [61], and we confirmed differences in the determined IC_{50} values between isolate WBC6 (Assemblage AI) and the Assemblage B isolates (GS/M, P424/A5). Notably, we tested all components with WBC6 and GS/M isolates maintained in two laboratories and encountered only minimal differences in the results, highlighting the robustness of our findings. The observed variability in IC_{50} for both MTZ and β-lapachone between Assemblages and laboratory isolates batch is consistent with data reported for MTZ in WBC6 by different authors, with 48-hour IC_{50} ranging from 2.1 to 8.5 μM (as measured by ATP content assay) [43,62–64]. Assessment of drug safety on an appropriate *in vitro* cellular model is a fundamental step in the process of selecting the most promising drug candidates to progress to pre-clinical *in vivo* test. Our results suggest that Caco-2 and MDCK epithelial cell lines might not be appropriate, despite their common use for the pharmaceutical evaluation of drug permeability [65]. While TH did not exhibit significant cytotoxic effects in either cell line at any time point, with a SI >100, TD and, in particular, β-lapachone were shown to be cytotoxic. The cytotoxic effect of β-lapachone has indeed been observed in various human carcinoma cell lines, such as oral squamous cell carcinoma, hepatocellular carcinoma, and gastric and colon adenocarcinoma [66,67]. Similar to findings in several epithelial cell lines [68], no prior evidence of β-

lapachone cytotoxicity has been reported for MDCK [69]. However, different studies have also shown inconsistent results across various cell lines [70,71]. Few studies have reported on testing of *T. avellanedae* alcoholic extracts both *in vitro* and *in vivo*, even if with contradictory results. While *T. avellanedae* ethanol extract showed no cytotoxicity in macrophage-like (RAW264.7) and chondrosarcoma (SW1353) cell lines [72], a methanol extract was toxic to human tumour cell lines but not to healthy cells [73]. Lemos *et al.* [74] (2012) reported increased DNA damage in liver cell nuclei of rats treated with a hydroalcoholic extract compared to controls. In contrast, de Miranda *et al.* [75] found that an aqueous extract of *T. avellanedae* inner bark was not acutely toxic in mice at doses up to 5000 mg/kg. The discordant observation of these studies regarding toxicity can be linked to variation in incubation time with the compound, concentration or dosage applied, variation in metabolite composition due to plant species and geographical location, or extraction methods [71].

The use of organoids is becoming a new standard for drug screening, as they can replicate more physiological conditions or even allow for personalized medicine when derived from patient [76]. Here, we confirmed that a well-established and characterised human duodenal ODM infection model [40–42] provides robust evidence of the safety of all the compounds at any of the tested concentrations, with no significant cytotoxicity over a 48-hour period and without disrupting intestinal epithelial cell integrity, as indicated by no significant reduction of TEER measurements. In the view of better compliance with the 3R approach, we have also shown that human duodenal ODM infected with *G. duodenalis* can simultaneously provide valuable information on both drug efficacy against the parasite and safety for host cells, minimizing confounding effects of infection on drug effect. Indeed, the ODM were infected with a MOI of 1 ($2x10^5$), as this MOI did not show any barrier disruption or TEER decrease in a previous study [40]. Both β-lapachone and TH have demonstrated effective reductions in *G. duodenalis* trophozoites, showing dose-dependent activity without any reduction in TEER or barrier destruction observed in immunofluorescence analysis. In comparison to tests performed in axenic *in vitro* conditions (Table 2), the IC_{50} values for WBC6 trophozoites treated with both β-lapachone (7.2 µM; 95% CI 3.7 to 14.4) and TH (1.4 mg/ml; 95% CI 1.2 to 1.6) (Supplementary Figure S5) were slightly higher, which may be due to

assay-related differences (e.g., medium volume, apical treatment selection in the trans-well assay) or possible metabolization of the compounds by host cells. Furthermore, the higher IC_{50} values observed in the ODM model suggest an increased tolerance of trophozoites in the presence of host cells compared to axenic culture. This finding aligns with previous research suggesting that *G. duodenalis*, and other intestinal parasites, exhibits enhanced viability when interacting with host cells [77,78]. This protective effect is likely due to the host-parasite interactions, which may provide a more supportive environment for parasite survival [78].

Conclusions

In conclusion, our *in vitro* results highlight the therapeutic potential of *T. avellanedae* against *G. duodenalis*. This natural extract and β-lapachone warrant further investigation as a novel antigiardial therapeutic; an in-depth exploration of their mechanism of action could unveil valuable therapeutic strategies that would contribute to the fight against drug failures and help prevent drug resistance. Additionally, this study marks the innovative utilisation of stem cell based ODM technology for evaluating antigiardial treatments, as it allows co-culturing and assessment under more physiological conditions than previous model using immortalised cell lines.

CRediT authorship contribution statement

Giulia Rigamonti: Writing – original draft, Writing – review & editing, Investigation, Methodology, Visualization. **Fabrizia Veronesi:** Writing – original draft, Conceptualization, Supervision. **Elisabetta Chiaradia:** Writing – review & editing, Formal analysis. **Petra Gosten-Heinrich:** Investigation, Formal analysis, Resources. **Antonia Müller:** Investigation, Formal analysis, Methodology. **Leonardo Brustenga:** Writing – review & editing**. Stefano de Angelis:** Resources**. Alessia Tognoloni:** Writing – review & editing, Investigation. **Riccardo De Santo:** Investigation, Methodology**,** Writing – original draft. **Christian Klotz:** Writing – review & editing, Conceptualization, Formal analysis, Visualization, Supervision. **Marco Lalle:** Writing – review & editing, Investigation, Conceptualization, Visualization, Supervision.

Declaration of competing interest

Giulia Rigamonti, Fabrizia Veronesi, Elisabetta Chiaradia, Petra Gosten-Heinrich, Antonia Müller, Leonardo Brustenga, Alessia Tognoloni, Riccardo De Santo, Christian Klotz and Marco Lalle declare no competing interest concerning the research, authorship, publication of this article and/or financial and personal relationships that could inappropriately influence this work.
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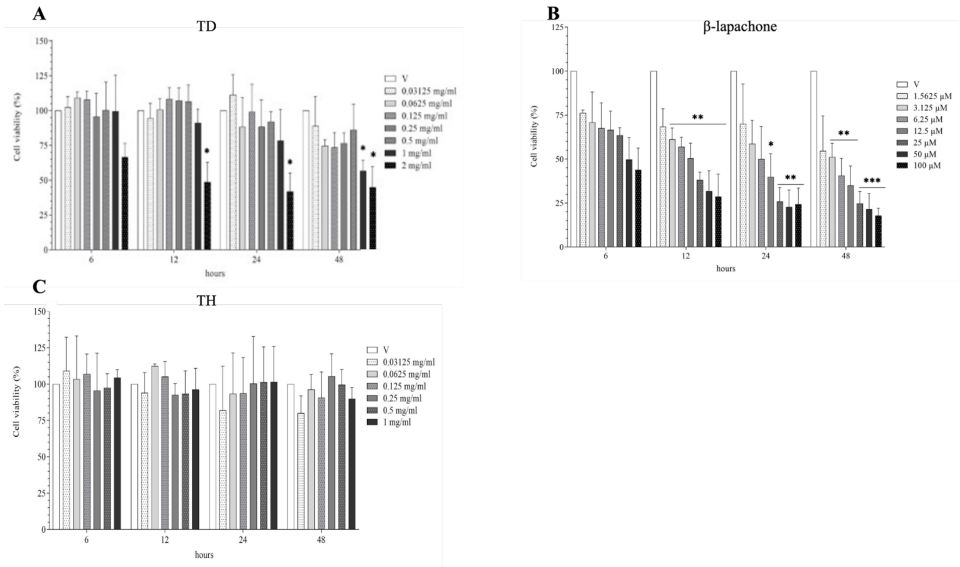
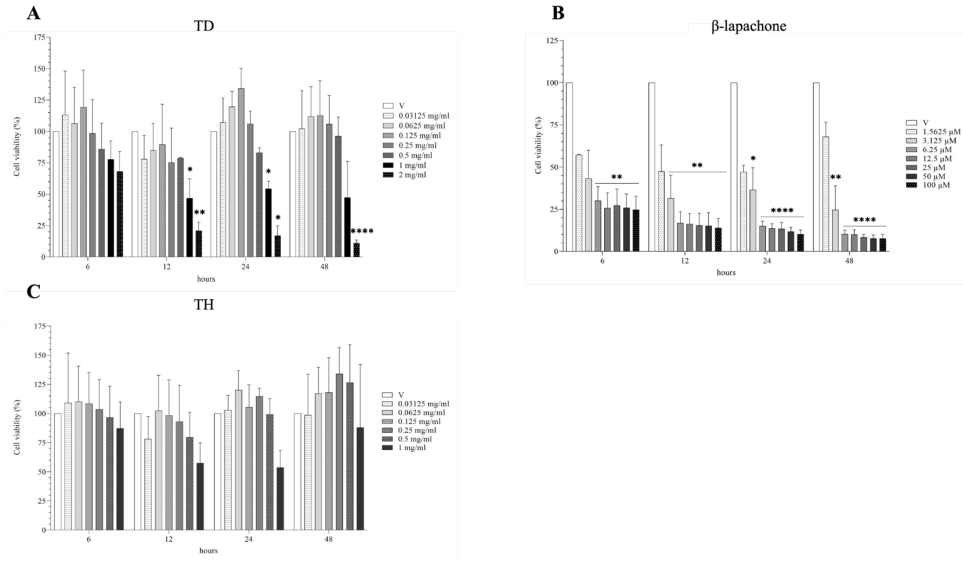
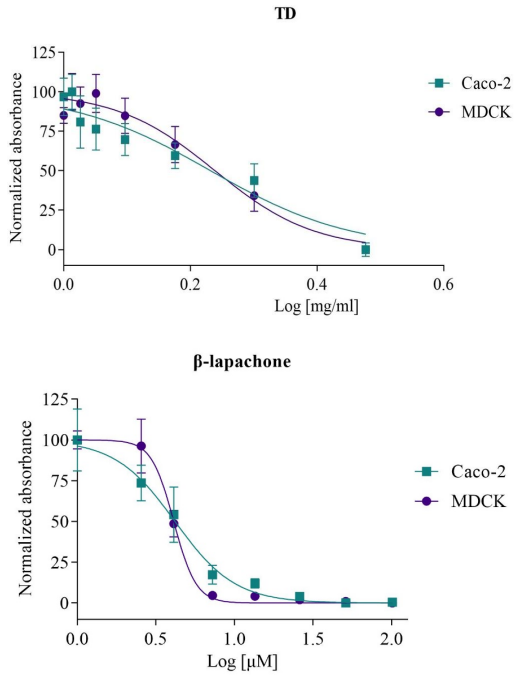
Supplementary Figures



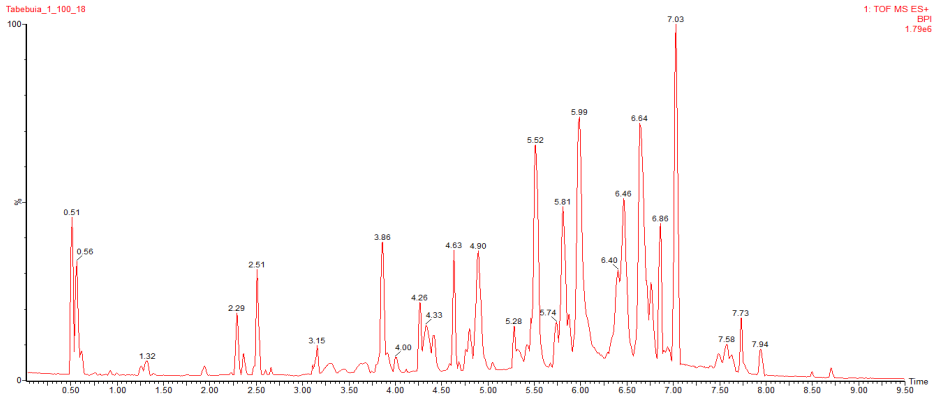
Supplementary Figure S1. Effects of different concentrations of *Tabebuia avellanedae* dry extract (TD) (A), β-lapachone (B), and *Tabebuia avellanedae* hydroalcoholic extract (TH) (C) on Caco-2 cell viability after 6, 12, 24, and 48 hours, tested using the CCK8 assay. The percentage of cell viability was calculated as the ratio between the optical density (OD) of the cells treated with specific compounds and the OD of the control cells. ***p<0.001, **p<0.01, *p<0.05 vs vehicle control (V), as indicated in figure.



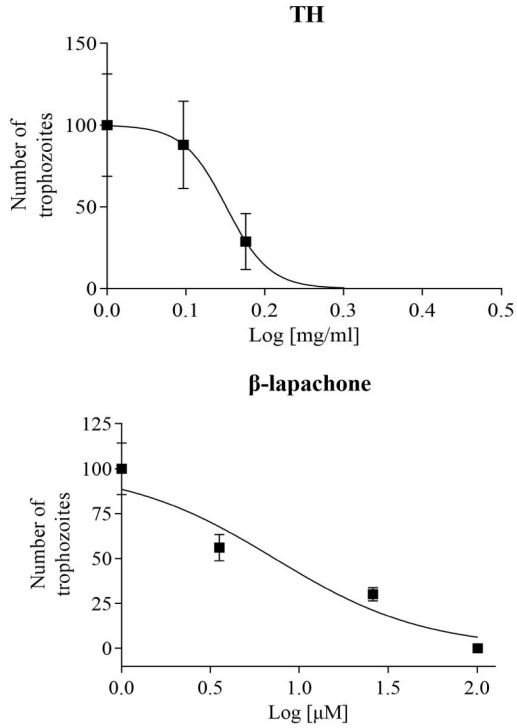
Supplementary Figure S2. Effects of different concentrations of *Tabebuia avellanedae* dry extract (TD) (A), β-lapachone (B), and *Tabebuia avellanedae* hydroalcoholic extract (TH) (C) on MDCK cell viability after 6, 12, 24, and 48 hours, tested using the CCK8 assay. The percentage of cell viability was calculated as the ratio between the optical density (OD) of the cells treated with specific compounds and the OD of the control cells. ***p<0.001, **p<0.01, *p<0.05 vs vehicle control (V), as indicated in figure.



Supplementary Figure S3. The 50% cytotoxic concentrations (CC_{50}) curves of *Tabebuia avellanedae* dry extract (TD) and β-lapachone against MDCK and Caco-2 cell lines at 48 hours. The CC_{50} values were determined using non-linear regression analysis in GraphPad Prism® version 9 (GraphPad Software, San Diego, USA).



Supplementary Figure S4. Base Peak Intensity (BPI) chromatogram of *Tabebuia avellanedae* hydroalcoholic extract (TH).



Supplementary Figure S5. The half maximal inhibitory concentrations (IC_{50}) curves of *Tabebuia avellanedae* hydroalcoholic extract (TH) and β-lapachone against *G. duodenalis* trophozoites cultivated on ODMs for 48 hours. The IC_{50} values were determined using non-linear regression analysis in GraphPad Prism® version 9 (GraphPad Software, San Diego, USA).

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Conclusions and future perspectives

Giardiasis represents a significant public health issue globally, with a considerable impact on both human and animal health. Despite therapeutic efforts, currently available drug treatments are associated with adverse side effects [1] and treatment failures [2–4], complicating disease management. In this context, the exploration of natural alternatives for the treatment of giardiasis has gained increasing interest [5]. In the present PhD Thesis, we assessed the *in vitro* activity of four natural plant extracts (*Morinda citrifolia*, *Panax ginseng*, *Tabebuia avellanedae*, and *Zingiber officinale*) and two isolated bioactive compounds (β-lapachone from *T. avellanedae* and 6-gingerol from *Z. officinale*) against *Giardia duodenalis* trophozoites, aiming to evaluate their potential as therapeutic agents. The initial results showed that, while the hydroalcoholic extract of *T. avellanedae* with β-lapachone exhibited significant anti-parasitic activity, the extracts from *M. citrifolia*, *P. ginseng*, and *Z. officinale*, as well as the bioactive compound 6-gingerol, showed no activity against *G. duodenalis* trophozoites. Consequently, we decided to focus our research on *T. avellanedae*.

Further *in vitro* results showed that both dry and hydroalcoholic extracts of *T. avellanedae* exhibited effective activity against zoonotic *G. duodenalis* trophozoite isolates (Assemblage AI and B), with similar 48-hour IC_{50} values across different isolates. Additionally, β-lapachone demonstrated strong antigiardial activity, consistent with previous studies [6], and outperformed metronidazole, showing slightly lower IC_{50} values for each isolate. These findings suggest that both *T. avellanedae* extracts and β-lapachone could serve as promising therapeutic options for the treatment of giardiasis. The potential efficacy of these compounds may lie in complex mechanisms, including effects on the redox state and the generation of reactive oxygen species (ROS), already demonstrated for another naphthoquinone tested against *G. duodenalis* [7], but investigating the mechanisms of action of β-lapachone and other identified metabolites, along with their interactions with *G. duodenalis* biology, will be essential to develop targeted treatments that can prevent drug resistance.

An innovative aspect of this research was the use of human intestinal organoid monolayers (ODMs) model infected with *G. duodenalis* trophozoites to test the safety and efficacy of the compounds, which proved

to be more physiologically relevant compared to traditional *in vitro* models, such as MDCK and Caco-2 [8,9]. Results from the *in vitro* cytotoxicity tests showed that none of the compounds tested reduced epithelial barrier integrity, suggesting that the *T. avellanedae* extract and β-lapachone are safe for host cells and do not cause significant damage at the tested concentrations. However, the higher IC_{50} values observed in the organoid model compared to axenic cultures suggest that host-cell interactions may influence the response of the trophozoites [10].

The results obtained confirm the therapeutic potential of the hydroalcoholic extract of *T. avellanedae* and its active compound, β-lapachone, as promising antigiardial agents. Their efficacy *in vitro*, combined with the absence of cytotoxic effects on host cells in more physiologically relevant models, such as ODMs, suggests that these compounds could provide a viable alternative for treating giardiasis, particularly considering the challenges posed by treatment failure with conventional drugs.

Future perspectives for the use of these natural compounds include exploring the pharmacokinetics and pharmacodynamics of the compounds *in vivo*, as well as its potential interactions with other existing therapies to improve overall treatment effectiveness. Clinical trials in infected pets could also provide valuable insights into the adoption of these therapeutic options for treating giardiosis in animals.

Finally, another promising avenue for future research is the exploration of *T. avellanedae* in combination therapy. Given the numerous advantages of combination treatments over monotherapy, combining different therapeutic agents can enhance efficacy, reduce the risk of resistance, and potentially minimize side effects, offering a more robust approach to treatment [11,12]. This could lead to improved clinical outcomes, particularly in complex conditions where single-drug therapies may be less effective.

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ADDENDUM

List of publications

1. Morganti G, **Rigamonti G**, Brustenga L, Calgaro V, Angeli G, Moretta I, Diaferia M, Veronesi F. Exploring similarities and differences between *Toxoplasma gondii* and *Neospora caninum* infections in dogs. Vet Res Commun. 2024 Dec;48(6):3563-3577. doi: 10.1007/s11259-024-10549-z.
2. Di Cesare A, Veronesi F, Crisi PE, Colombo M, Morelli S, Vignoli M, **Rigamonti G**, Iorio R, Tielemans E, Beugnet F, Gamblin C, Traversa D. Clinical efficacy of a parasiticide formulation containing eprinomectin, esafoxolaner and praziquantel (NexGard® Combo) in the treatment of natural feline aelurostrongylosis and troglostrongylosis. Vet Parasitol. 2024 Jul 22;331:110271. doi: 10.1016/j.vetpar.2024.110271.
3. Morganti G, **Rigamonti G**, Marchesi MC, Maggi G, Angeli G, Moretta I, Brustenga L, Diaferia M, Veronesi F. *Toxoplasma gondii* and *Neospora caninum* infection in epileptic dogs. J Small Anim Pract. 2024 Aug;65(8):631-636. doi: 10.1111/jsap.13735.
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6. Brustenga L, Franciosini MP, Diaferia M, **Rigamonti G**, Musa L, Russomanno BL, Veronesi F. Parasitological Survey in European Brown Hare (*Lepus europaeus Pallas, 1778*) Breeding Facilities in Southern Italy. Pathogens. 2023 Jan 29;12(2):208. doi: 10.3390/pathogens12020208.
7. Morganti G, Miglio A, Moretta I, Misia AL, **Rigamonti G**, Cremonini V, Antognoni MT, Veronesi F. Retrospective Longitudinal Survey on

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8. Gazzonis AL, Morganti G, Porcellato I, Roccabianca P, Avallone G, Gavaudan S, Canonico C, **Rigamonti G**, Brachelente C, Veronesi F. Detection of Leishmania spp. in Chronic Dermatitis: Retrospective Study in Exposed Horse Populations. Pathogens. 2022 May 31;11(6):634. doi: 10.3390/pathogens11060634.

Participation at international and national congresses

Oral presentations:

1. **Rigamonti G**, Lalle M, Klotz C, de Angelis S., Tognoloni A., Brustenga L., De Santo R., Chiaradia E., Veronesi F. "*In vitro* efficacy of Red Lapacho (*Tabebuia avellanedae*) against *Giardia duodenalis"*. 14th European Multicolloquium of Parasitology (EMOP), Wrocław, Poland, August 26-30, 2024.
2. **Rigamonti G**, Lalle M, Klotz C, de Angelis S, Tognoloni A, Brustenga L, De Santo R, Chiaradia E, Veronesi F. "*In vitro* efficacy of Red Lapacho (*Tabebuia avellanedae*) against *Giardia duodenalis"*. XXXIII Congresso Nazionale della Società Italiana di Parassitologia, Padova, June 18-21, 2024.
3. **Rigamonti G**., Diaferia M., Moretta I., Lepri E. "Un caso di ascaridiosi aberrante in un agnello" XXIV Congresso SIPAOC, Viterbo, February 22-24, 2023.
4. **Rigamonti G**., Gazzonis A., Morganti G., Roccabianca P., Avallone G., Porcellato I., Gavaudan S., Brachelente C., Veronesi F. "Detection of *Leishmania* spp. in chronic dermatitis: retrospective study in exposed horse populations" 75° Convegno SISVET, Lodi, June 15-18, 2022.

Poster presentation:

1. Brustenga L, **Rigamonti G**, Moretta I, Morganti G, Calgaro V, Diaferia M, Lepri E, Lucentini L, Veronesi F. "Urban wildlife, a spiky issue: first detection of *Giardia duodenalis* in Italian European hedgehogs (*Erinaceus europaeus*). 77° Convegno SISVET, Parma, 12-14 June 2024.