



RESEARCH ARTICLE

Antiparasitic activity of lipid extracts from the subantarctic macroalgae *Iridea cordata* against *Trichomonas vaginalis*

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Highlights

- Lipid extracts from *Iridea cordata* reported a cytotoxic effect against 100% of trophozoites at a final concentration of 600 µg/mL with an IC₅₀ of 300 µg/mL.
- Lipid extracts from *Iridea cordata* did not show cytotoxicity on HMVII cells and VERO cells.
- Lipid extracts from *Iridea cordata*, modified the expression levels of genes that were important for the metabolism of *T. vaginalis*.

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KEYWORDS

Natural extracts,
Anti-protozoan,
Trichomonacidal.

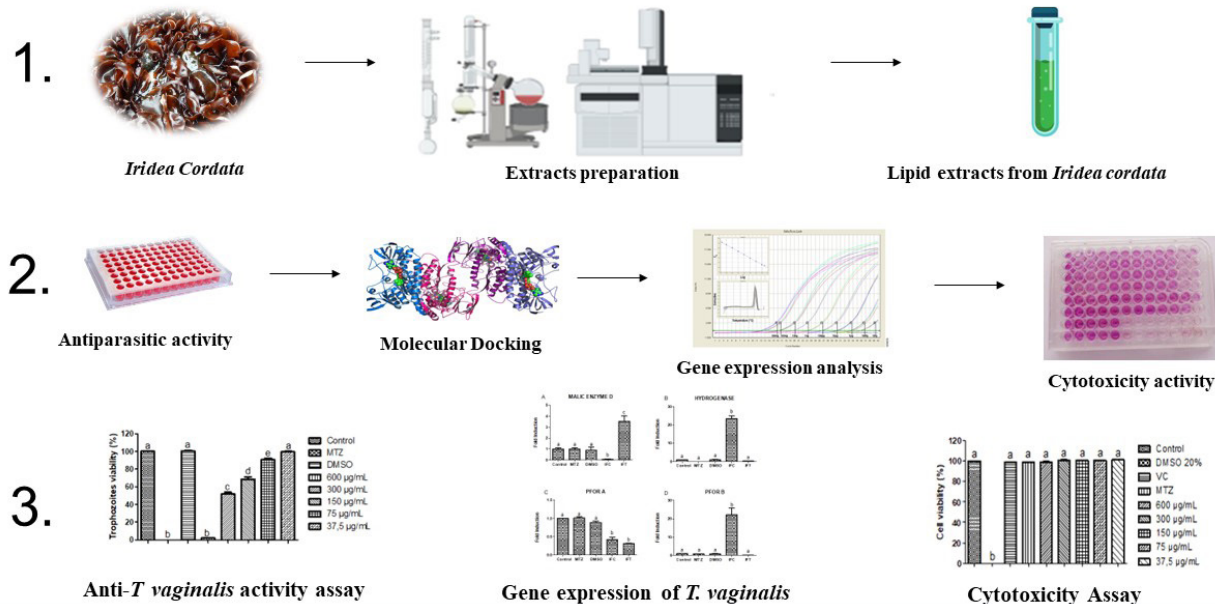
Abstract: In this study, we demonstrate the promising antiparasitic activity of natural extracts as an alternative treatment for trichomoniasis. We evaluated the *in vitro* and *in silico* antiparasitic activity of *Iridea cordata* extracts, obtained in two distinct development phases: *Iridea cordata* tetrasporophyte phase (IFT) and *Iridea cordata* cystocarp phase (IFC). To determine the minimum inhibitory concentration (MIC) and 50% inhibitory concentration (IC₅₀), we tested five concentrations of the extracts against *Trichomonas vaginalis* (ATCC 30236). To gain insights into the mechanisms underlying the antiparasitic activity and possible adverse effects, the extracts were subjected to cytotoxicity assays in VERO and human vaginal epithelial (HVMII) cells, gene expression analyses, and their components' interactions with *T. vaginalis* proteins were analyzed through molecular docking. In the *in vitro* biological assay, IFT and IFC exhibited a MIC of 600 µg/mL while showing an IC₅₀ of 150 µg/mL and 300 µg/mL and inhibiting 80% and 97% of *T. vaginalis* trophozoites, respectively. Importantly, no cytotoxic effects were observed on VERO and HMVII cells for IFC and IFT at 600 µg/mL, indicating their safety. IFC and IFT induced significant differences in gene expression compared to the negative control, DMSO, and metronidazole, suggesting their potential modulation of *T. vaginalis* genes. Moreover, *in silico* analysis revealed that constituents of both extracts interacted, with significant free-binding energy, with proteins that are important for *T. vaginalis* survival. Overall, this study provides evidence of the antiparasitic activity of *Iridea cordata* extracts against *T. vaginalis* and supports further evaluation of its extracts as a promising treatment for trichomoniasis.

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Graphical Abstract



Introduction

Trichomoniasis is among the most prevalent non-viral sexually transmitted diseases worldwide (Alves et al., 2020). The etiological agent of this disease is *Trichomonas vaginalis*, a unicellular flagellate parasitic protozoan that infects the human urogenital region (Aquino et al., 2020). During the process of infection, the parasite adheres to genital epithelial cells (Mercer & Johnson, 2018), adapting quickly and performing the phagocytosis of various cells while also evading the immune system, moving through cell junctions, and colonizing the lower epithelial layers of the host (Ryan et al., 2011).

The symptoms of trichomoniasis in women include vaginal discharge, burning sensation while urinating, vulvar irritation, and abdominal pain (Nemati et al., 2018). In men, the disease is usually asymptomatic, and when symptomatic, dysuria and urethral discharge are observed (Patel et al., 2018). Metronidazole (α -hydroxyethyl-2-methyl-5-nitroimidazole) is the most commonly used therapeutic agent to treat trichomoniasis (Ghosh et al., 2018). Metronidazole (MTZ) is the main compound of the nitroimidazole family and acts mainly on *T. vaginalis* DNA (Mendes et al., 2020).

Despite its low cost and proven efficacy, clinical treatment failures associated with side effects, such as allergic reactions, fever, swelling, and erythema, are observed (Singh et al., 2019). In addition, there is increasing resistance to MTZ among parasites (Ozpinar et al., 2019). Therefore, the pursuit of new therapies applying natural products to effectively control parasites and minimize adverse effects holds significant relevance in combating trichomoniasis (Chellan et al., 2019).

Recently, natural marine products have raised scientific interest due to their pharmacological effects, which arise from their bioactive constituents (Pacheco et al., 2018). In this regard, macroalgae, a diverse group of organisms encompassing nearly 20,000 identified species, are considered remarkable candidates for scientific research (Mekinić et al., 2019). In addition, macroalgae are organisms that can grow in adverse conditions (Ho, 2020), as their adaptability allows them to survive in inhospitable ecosystems (Jofre et al., 2020). These organisms have been found to produce complex molecular compounds in response to environmental stimuli. These compounds exhibit the ability to interact with various molecular receptors within cells of other organisms, leading to the inhibition or promotion of specific biological effects (Besednova et al., 2021). Therefore, such compounds hold potential for therapeutic applications. The bioactive compounds generated from macroalgae have already demonstrated several pharmacological effects of interest (Alkaabi & Almayali, 2018), such as immunosuppressant, antioxidant, anti-inflammatory, antiproliferative, antibacterial (Sánchez-Camargo et al., 2016), antidiabetic, anticancer (Deng et al., 2020), hepatoprotective, anticholinergic, antihistamine, and antifungal (Martínez-Hernández et al., 2018). Thus, these marine organisms represent novel sources of bioactive compounds and warrant further investigation of their antiparasitic effects (Álvarez-Bardón et al., 2020).

Therefore, in this study, we investigated the antiparasitic activity of lipid extracts from two distinct developmental phases, cystocarp and tetrasporophyte, of the subantarctic macroalgae *Iridea cordata* against *T. vaginalis* trophozoites.

Materials and methods

Sampling

Iridea cordata specimens (tetrasporophyte and cystocarp phases) were manually collected from the Punta Arena region in January 2017 (Latitude: -53.1667, Longitude: -70.9333 53° 10' 0" South, 70° 55' 60" West). After collection, the samples were washed and morphologically identified. Subsequently, they were lyophilized, pulverized, and stored in dark plastic bags within a desiccator to ensure protection from heat, light, and humidity before analysis. After that, the macroalgae species were identified at the Herbarium of the Laboratory of Antarctic and Subantarctic Marine Ecosystems (LEMAS) at the University of Magalhães (UMAG) in Punta Arenas, southern Chile.

Fatty Acids extraction

The Fatty Acids (FAs) were extracted from the samples using a method described by Bligh & Dyer (1959). Briefly, a mixture containing 20 mL of methanol, 10 mL of chloroform, 10 mL of a 1.5% aqueous sodium sulphate solution (w/v), and 1 g of macroalgae biomass was constantly stirred at room temperature for 30 min. Subsequently, the samples were placed in conical tubes and centrifuged at 3000 rpm for 30 min. Then, the lower layer of the organic phase was retrieved and dried under reduced pressure. The procedure was performed in triplicate (n = 3).

Derivatization

FAs derivatization was performed according to the method described by Moss et al. (1974). Briefly, the extracted material was refluxed with a 0.5 M methanolic solution of sodium hydroxide at 100 °C for 5 min. Then, 5 mL of a 14% methanolic solution of boron trifluoride was placed in the sample while maintaining the reflux at 100 °C for another 5 min. Subsequently, the system was cooled with 3 mL of a saturated aqueous solution of sodium chloride, and 20 mL of n-hexane was added to the solution. The solution was then transferred to a separation funnel to separate the upper organic phase, which was then filtered with anhydrous sodium sulphate and dried under reduced pressure. The procedure was performed in triplicate (n = 3).

Instrumentation and quantification

After FAs extraction and derivatization, samples were diluted in n-hexane and introduced in a GC-FID model GC-2010 (Shimadzu, Kyoto, Japan), using nitrogen as the carrier gas and an SP-2560 capillary column (100 m x 0.25 mm x 0.2 µm) from Supelco (Bellefonte, USA). The temperature was initially set at 140 °C then increased at a rate of 4 °C per minute until it reached 240 °C, which was maintained for 10 min. Thus, the total run was 40 min. The injector was maintained at 260 °C while the injections were performed in the split mode (1:100). To identify and quantify the FAs, samples were compared to a FAME 37-Mix standard using the GC Solution software (Shimadzu, Kyoto, Japan).

Chemicals and standards

Methanol, chloroform, sodium chloride, anhydrous sodium sulphate, and sodium hydroxide were purchased from Labsynth (Diadema, Brazil), while HPLC-grade n-hexane was acquired from JT Baker (Phillipsburg, USA). The methanolic solutions of boron trifluoride and nonadecanoic methyl ester (C19:0) were purchased from Sigma-Aldrich (St. Louis, USA). The standard 37-mix of fatty acid methyl esters was obtained from Supelco (Bellefonte, USA). All standards and chemicals were of analytical grade.

Anti-*T. vaginalis* assay

In this study, we used *T. vaginalis* isolate 30236, which was obtained from the American Type Culture Collection (ATCC). This isolate is known to be susceptible to metronidazole. To cultivate the trophozoites, axenic conditions were maintained, and they were grown in a trypticase-yeast extract-maltose (TYM) medium without agar (pH 6.0). The TYM medium was supplemented with 10% sterile bovine serum (SBS), previously inactivated at 56 °C. Trophozoites were then incubated at 37 °C (Diamond, 1957).

To evaluate the anti-*T. vaginalis* activity of the cystocarp phase (IFC) and tetrasporophyte phase (IFT) lipid extracts, we applied the methodology described by Sena-Lopes et al. (2017). IFC and IFT extracts were tested in cultures with at least 95% viability based on their motility, morphology, and the trypan blue exclusion test (0.4%), which was performed by optical microscopy at 400× magnification. All tests were performed in 96-well microtiter plates (Cral®). To evaluate the MIC and IC₅₀ against *T. vaginalis*, we followed (Hübner et al., 2016) methodology with some adaptations. The parasites were seeded at an initial density of 2.6 × 10⁵ trophozoites/mL of TYM and incubated with IFC and IFT lipid extracts previously diluted in dimethylsulfoxide (DMSO). Subsequently, the lipid extracts were assessed at various concentrations: 600, 300, 150, 75, and 37.5 µg/mL. The experiment included three control groups: a negative control consisting solely of trophozoites, a positive control containing 100 µM MTZ (Sigma-Aldrich), and a control to account for the vehicle used to solubilize the IFC and IFT extracts, which consisted of 0.6% DMSO. The plates were then incubated at 37 °C in 5% CO₂ for 24 h. After incubation, an aliquot of trophozoites and trypan blue (0.4%) (1:1, v/v) was mounted in a Neubauer chamber to determine the MIC, considering the motility, morphology, and viability of the trophozoites. To confirm the MIC, the parasites were treated with both lipid extracts and controls for 24 h. After that, treatment and control samples were incubated in tubes containing 1.5 mL of fresh TYM medium supplemented with SBS and antibiotics at 37 °C. The trophozoite counts were then conducted every 24 h for 96 h using a Neubauer chamber and a preparation of trophozoites with trypan blue solution. To determine the IC₅₀, the data obtained were analyzed using GraphPad Prism version 7.0 software.

For a more accurate profile of the anti-*T. vaginalis* activity exerted by the lipid extracts, a kinetic growth curve was determined. The 96-well plates were prepared as described above and incubated with the lipid extracts at their MIC.

Growth analysis was performed at 1, 6, 12, 24, 48, 72, and 96 h by the trypan blue dye exclusion test (0.4%). All assays were performed independently in triplicate, and the results were expressed as the percentage of viable trophozoites compared to untreated parasites.

Cytotoxicity test

The cytotoxicity was assessed as described by (Navarrete-Vázquez et al., 2015) using two distinct cell lines: VERO cells and vaginal epithelial cells (HMVII). A suspension of 2.5×10^4 viable cells was incubated in a 96-well plate for 24 h. The VERO and HMVII cells were incubated in DMEM and RPMI-1640 mediums, respectively, enriched with 10% Fetal Bovine Serum (FBS) and 2% antibiotic-antimycotic at 37 °C, 5% CO₂, and 95% humidity for 24 h. When the cells reached 75% confluence, the medium was replaced, and the cells were treated with the IFC and IFT lipid extracts at 600, 300, 150, 75, and 37.5 µg/mL for another 24 h at 37 °C and 5% CO₂.

Four controls were used in the test: untreated cells as the negative control, a control to account for the vehicle used to solubilize the extracts (0.6% DMSO), and 100 µM MTZ and 20% DMSO as positive controls. After incubation, the medium was removed, and 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide solution (5 mg/mL) was added to each well, followed by incubation for three hours at 37 °C in a 5% CO₂ atmosphere. Then, the MTT solution was removed, and 100 µL of DMSO was added to each well to solubilize the formazan crystals. Then, the reduction of MTT to formazan, which is directly proportional to the number of living cells, was observed in a microplate reader at 492 nm. The results were expressed as the percentage of viable cells compared to untreated cells. All observations were validated by at least three independent experiments in triplicate.

T. vaginalis gene expression

To evaluate changes in *T. vaginalis* gene expression after treatment, we isolated the total RNA of trophozoites (10^7 cells/sample) previously incubated with the MIC of the lipid extracts. To establish a basis for comparison, we also extracted total RNA from three control groups: untreated

cells as negative control, cells treated with 0.6% DMSO, and cells treated with 100 µM MTZ. The RNA isolation was performed using Trizol reagent (Invitrogen) following the manufacturer's instructions. The cDNA was synthesized from 0.5 µg of RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosynthesis), according to the manufacturer's protocol. Real-time PCR was performed on a 20 µL reaction mixture containing 10 µL of SYBR Green PCR Master Mix (Applied Biosystems, UK), 300 nM of primers, 1 µL of cDNA, and RNase-DNase free water. The PCR conditions were initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The melting curve was analyzed following the cycling protocol of 95 °C for 15 s, 55 °C for 15 s, and 95 °C for 15 s.

The real-time PCR reactions were performed on the Stratagene Mx3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) using the primers described in Table 1. The actin α gene was used as a normalizer in all the analyses.

T. vaginalis protein and ligand structures and molecular docking analysis

Considering the promising *in vitro* antiparasitic activity of IFC and IFT against *T. vaginalis*, we assessed other possible biological targets using molecular docking analyses. The protein structures for *T. vaginalis* triphosphate isomerase (TvTPI), lactate dehydrogenase (TvLDH), methionine gamma-ligase (TvMGL), purine nucleoside phosphorylase (TvPNP) were obtained from Protein Data Bank (PDB: 1E5E, 1Z36, 3QST, and 5A1T, respectively), whereas those of papain-like cysteine protease (TvCP2), thioredoxin reductase (TvTrxR), and cathepsin-like cysteine protease (TvCPCA1) were predicted using I-TASSER (Yang et al., 2020), based on their primary sequence obtained from UniProt (UniProt: Q27107, Q8IEV3, and Q6UEJ4). The structures of ligands were obtained in the SDF and SMILES format from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The ligands were converted from the SDF to the PDB format using the program OpenBabel (O'Boyle et al., 2011), and the results were then processed using the script "prepare_ligand4.py" from Autodock Tools to produce PDBQT files for the docking procedure (Morris et al., 2009).

Table 1. Primers used for qRT-PCR analysis of *Trichomonas vaginalis* ATCC 30236 treated with extracts from *Iridea cordata* tetrasporophyte and cystocarp phases.

Gene	Primer	Sequence (5' - 3')	GenBank Access Number	Reference
Actin α	F R	TCACAGCTCTTGCTCCACCA AAGCACTTGCGGTGAACGAT	KF747377.1	(Santos, et al., 2015)
PFOR A	F R	CGGCTACGGTATGTTCAAGG TCCTTGCTCTGATCCCAAC	U16822	(Mead et al., 2006)
PFOR B	F R	CTGCAAGCTCCTTACACAGC AAGAGGGAGTTAGCCCAAGC	U16823	(Mead et al., 2006)
Malic enzyme(D)	F R	CATCTGTTAGCCTCCCAGTCC ACGAGCAGCTTGTTTCATCCT	U16839	(Mead et al., 2006)
Hydrogenase	F R	TGCACACGAAAGAAGGATGA TCGCATGGTGTATCTGGTAA	U19897	(Mead et al., 2006)

The receptor files in PDB format were then analyzed using the program COACH (Yang et al., 2013) to identify potential binding pockets and processed using the script “prepare_receptor4.py”, also from Autodock Tools, and converted to the PDBQT format. The processed ligands and receptors were then docked on the predicted binding sites using the program Autodock Vina (Trott & Olson, 2010).

Statistical analysis

The treatment and control groups were compared using a one-way analysis of variance (ANOVA), with a p-value of < 0.05 indicating statistical significance. Concomitantly, Tukey’s test was applied to compare the means of different

pairs of treatments. The statistical analysis was performed using the software GraphPad Prism version 5.0.

Results

Chemical components of lipid extracts

The constituents of *Iridea cordata* lipid extracts were identified using a gas chromatography-flame ionization detector (GC-FID) (Table 2). The analyses showed that the IFC and IFT lipid extracts contained 31.87 and 30.08% saturated fatty acids, 16.57 and 13.74% monounsaturated fatty acids, and 51.56 and 56.18% polyunsaturated fatty acids, respectively.

Table 2. Fatty acids in the cystocarp and tetrasporophyte phases of the lipid extracts of *Iridea cordata* were identified by a gas chromatography-flame ionization detector (GC-FID).

Fatty acids	<i>Iridea cordata</i>	
	Cystocarp (%)	Tetrasporophyte (%)
Lauric acid C12:0	0.65	0.46
Myristic acid C14:0	2.64	2.55
Pentadecanoic acid C15:0	0.55	-
Palmitic acid C16:0	23.92	24.71
Heptadecanoic acid C17:0	0.48	-
Stearic acid C18:0	3.1	2.36
Lignoceric acid C24:0	0.53	-
Palmitoleic acid C16:1	4.06	2.16
Oleic acid C18:1N9C	10.08	9.48
Erucic acid C20:1N9C	0.51	0.65
Nervous acid C24:1N9	0.77	0.54
Linolelaidic acid C18:2N6T	1.15	0.91
Linoleic acid C18:2N6C	2.2	1.19
Linolenic acid C18:3N3	4.52	3.71
Gamma linolenic acid C18:3N6	5.21	3.42
Cis-11.14 eicosadienoic acid C20:2	0.53	0.73
Cis-8.11.14 eicosatrienoic acid C20:3N6	2.17	2.16
Arachidonic acid C20:4N6	1.99	1.6
Cis-5.8.11.14.17 eicosapentaenoic acid C20:5N3	17.29	21.92

Anti-*T vaginalis* activity assay

The IFC and IFT lipid extracts at a concentration of 600 $\mu\text{g}/\text{mL}$ reduced the viability of trophozoites by 97 and 80%, respectively, after 24 h of exposure (Figure 1). This concentration was considered the MIC of these extracts.

The IC_{50} value was statistically established as 300 $\mu\text{g}/\text{mL}$ for IFC and 150 $\mu\text{g}/\text{mL}$ for IFT. In the negative control and 0.6% DMSO groups, as anticipated, the trophozoites were motile and did not show any staining when exposed to 0.4% trypan blue. Conversely, the positive control group (100 μM MTZ) exhibited diminished viability of the parasites, evident by their loss of motility and effective staining with 0.4% trypan blue after 24 h of exposure (Figure 1).

Regarding the kinetic growth curve, it was observed that exposure to 600 $\mu\text{g}/\text{mL}$ of IFC reduced the growth of trophozoites by 45% within 12 h, with no significant difference when compared to the MTZ positive control after 24 h of exposure. Moreover, exposure to 600 $\mu\text{g}/\text{mL}$ of IFT reduced the growth of trophozoites by 80% within 24 h, demonstrating persistent inhibitory action after this period (Figure 2).

Cytotoxicity Assay

The *in vitro* cytotoxicity of IFC and IFT was assessed by the MTT assay on two mammalian cell lines: African green monkey kidney epithelial cells (VERO) and vaginal epithelial cells (HMVII) (Figure 3).

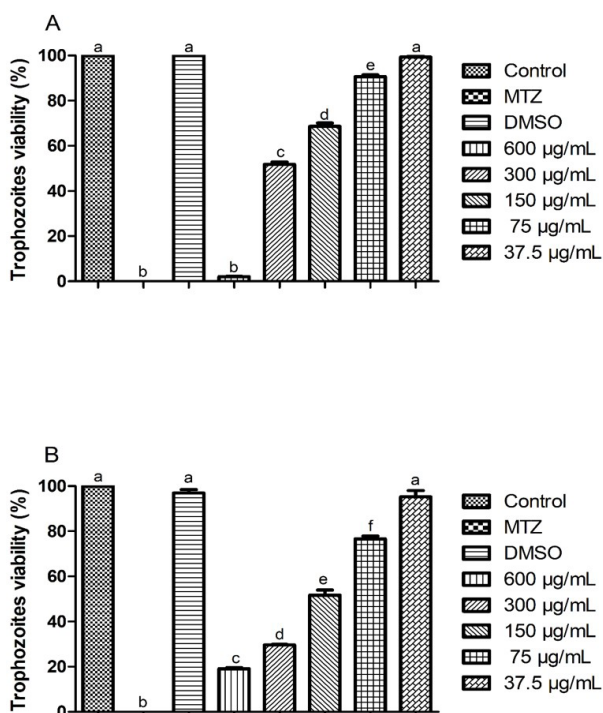


Figure 1. Determination of the MIC and IC_{50} for the lipid extracts of *Iridea cordata* cystocarp (IFC) (A) and tetrasporophyte (IFT) (B) phases against *Trichomonas vaginalis* ATCC 30236 after treatment with 600, 300, 150, 75, and 37.5 $\mu\text{g}/\text{mL}$ for 24 h. Dimethyl sulfoxide was used as a dilution vehicle (DMSO 0.6%), and 100 μM metronidazole (MTZ) as a positive control. Data represent the mean \pm standard deviation of at least three experiments, all in triplicate. Different letters indicate a significant difference ($p < 0.05$).

The results indicated that a 24-h exposure to IFC and IFT at their respective MIC, VERO cells exhibited cell viability of 99.69% and 99.29%, while HMVII cells showed 86.46% and 86.84% cell viability, respectively. As expected, the solubilization vehicle (0.6% DMSO) did not affect cell growth, while the positive control (20% DMSO) induced 100% cell mortality. The results indicated that both extracts were not cytotoxic to the target cells, even at higher concentrations (Figure 3).

T. vaginalis gene expression

A quantitative PCR was conducted to assess the transcription levels of malic enzyme D, hydrogenase, and pyruvate ferredoxin oxidoreductase (PFOR) type A and B after treating *T. vaginalis* cultures with IFC and IFT lipid extracts. Remarkably, both IFC and IFT demonstrated a distinct expression profile of these enzymes (Figure 4).

When *T. vaginalis* trophozoites were incubated with IFC, the mRNA expression for hydrogenase and PFOR B was upregulated ($p < 0.05$), whereas, in the case of IFT, the expression of these enzymes was not significantly affected. On the other hand, IFT induced upregulated expression of malic enzyme D, which was not significantly affected by IFC. Both IFC and IFT inhibited the expression of PFOR A. No significant induction or inhibition of these enzymes was observed in the groups treated with DMSO or MTZ.

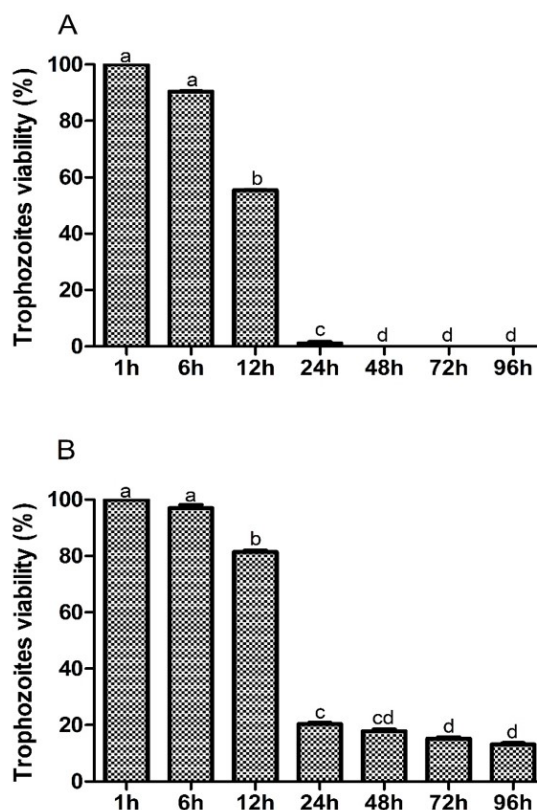


Figure 2. Kinetic growth curves of *Trichomonas vaginalis* 30236 isolate after 1, 6, 12, 24, 48, 72, and 96 h of treatment with 600 $\mu\text{g}/\text{mL}$ IFC (A) and IFT (B). Data represent the mean \pm standard deviation of at least three experiments, all in triplicate. Different letters indicate a significant difference ($p < 0.05$).

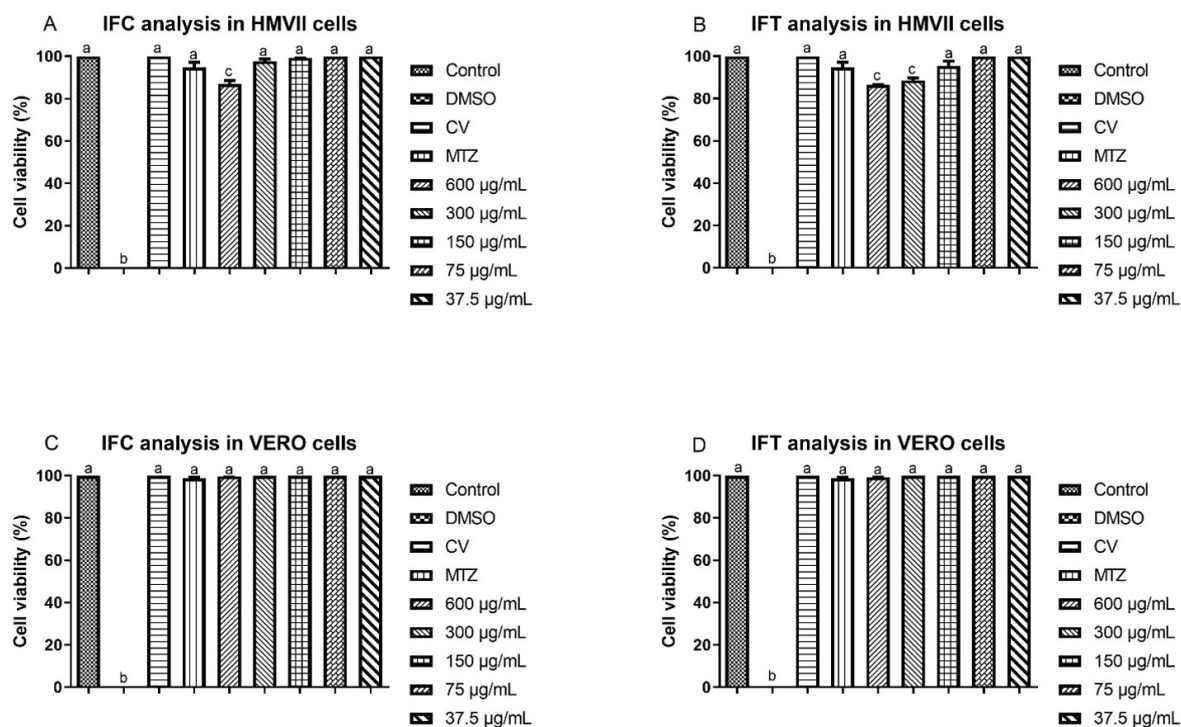


Figure 3. *In vitro* cytotoxicity assay of lipid extracts from *Iridea cordata* cystocarp (IFC) and tetrasporophyte (IFT) phases at 600 µg/mL (MIC) against human vaginal epithelial cells (HMVII) and VERO cells. (A) IFC + HMVII cells, (B) IFT + HMVII cells, (C) IFC + VERO cells, and (D) IFT + VERO cells. Cell proliferation was investigated by MTT assay after 24 h of exposure to IFC and IFT at 600 µg/mL. The control groups were as follows: negative control (untreated cells), positive control (20% DMSO), CV (dilution vehicle - 0.6% DMSO), and MTZ (100 µM metronidazole). Data are presented as the mean ± standard deviation of at least three independent experiments. Different letters indicate a significant difference between treatments at $p < 0.05$.

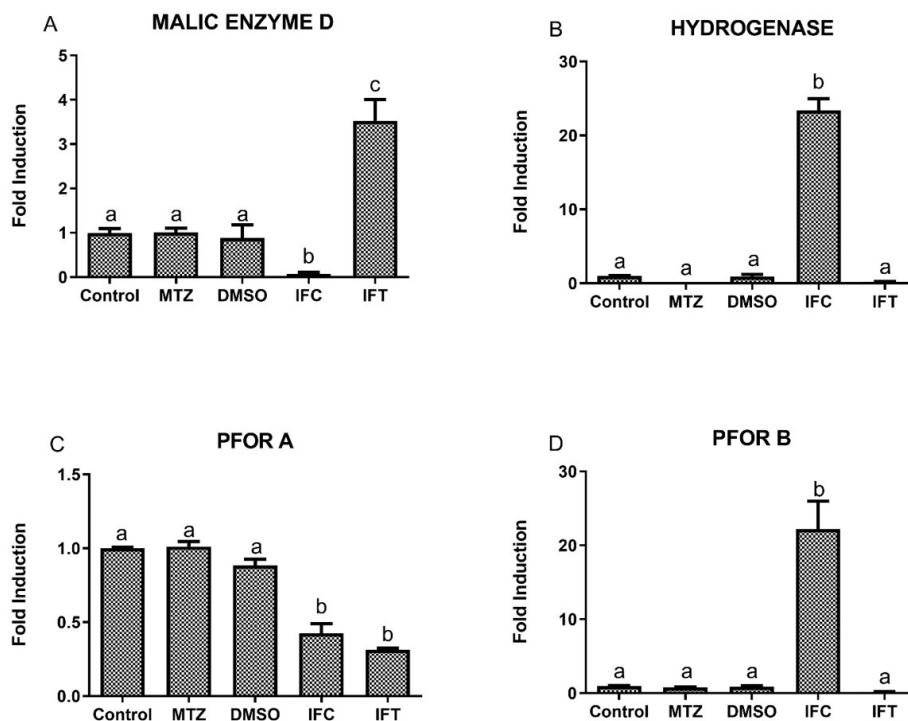


Figure 4. IFC and IFT lipid extracts at a concentration of 600 µg/mL alter gene expression in *Trichomonas vaginalis*. The gene expression profile of *Trichomonas vaginalis* of malic enzyme D (A), hydrogenase (B), pyruvate ferredoxin oxidoreductase (PFOR) type A (C) and pyruvate ferredoxin oxidoreductase (PFOR) type B (D) was determined by qRT-PCR. Data are expressed as the means ± standard deviation of at least three independent experiments. The controls used were negative control (untreated *T. vaginalis*), solubilization vehicle (DMSO), and MTZ (metronidazole).

Molecular docking of fatty acids and *T. vaginalis* enzymes.

To explore the potential antiparasitic activity of IFC and IFT extracts *in vitro*, additional targets were investigated to understand the interactions between the constituents of these extracts and *T. vaginalis* enzymes and their contribution to trichomonocidal activity.

The *in silico* analysis indicated that all constituent of both extracts exhibited the ability to interact with the target-binding sites that were analyzed. The most favorable binding free energy (-7.6 kcal/mol) was observed between TvMGL and oleic acid, followed by the interaction between eicosapentaenoic acid and TvLDH (-6.2 kcal/mol) (Table 3).

Furthermore, all the evaluated fatty acids showed significant levels of free energy when interacting with the TvLDH binding site, surpassing the levels observed for other targets. The molecular docking scores between TvCP2, TvTPI, TvCPCAC1, and TvTrxR were not significantly different. Overall, the results from this *in-silico* analysis of this study suggested an association between these proteins and the antiparasitic activity of IFC and IFT against *T. vaginalis*.

Discussion

Trichomoniasis is a sexually transmitted infection that significantly impacts public health worldwide, and it can have severe consequences in individuals with conditions such as cervical cancer, prostate cancer, and chronic cervicitis, among others (Trein et al., 2019). Moreover, the side effects associated with clinical treatment using metronidazole have resulted in low patient compliance and a decrease in the success of this therapeutic approach, leading to the emergence of resistant strains. Therefore, searching for alternative treatment approaches is crucial to combat trichomoniasis (Ziaei Hezarjaribi et al., 2019). In this study, we demonstrate the antiparasitic effect of lipid extracts from two developmental phases of *Iridea cordata* against *T. vaginalis* using *in silico* and *in vitro* assays.

The GC-FID technology has been widely used by the petrochemical and pharmaceutical industry, besides being described in the literature as one of the best tools for the characterization of fatty acids (Zhang et al., 2015).

Table 3. Docking scores (kcal/mol) for the *in silico* interaction between the fatty acids found in *Iridea cordata* lipid extracts (IFC and IFT) and *Trichomonas vaginalis* papain-like cysteine protease (TvCP2), triphosphate isomerase (TvTPI), lactate dehydrogenase (TvLDH), methionine gamma-ligase (TvMGL), cathepsin-like cysteine protease (TvCPCAC1), purine nucleoside phosphorylase (TvPNP), and thioredoxin reductase (TvTrxR). IFC, *Iridea cordata* cystocarp phase lipid extract. IFT, *Iridea cordata* tetrasporophyte phase lipid extract.

Fatty acids	<i>T. vaginalis</i> enzymes (targets)						
	TvCP2	TvTPI	TvLDH	TvMGL	TvCPCAC1	TvPNP	TvTrxR
Arachidonicacid	-3.6	-4.3	-5.4	-5.1	-4.5	-3.1	-4.8
Cis-8.11.14-Eicosatrienoic acid	-4.4	-3.5	-5.5	-4.6	-4.7	-3.2	-4.7
Eicosadienoicacid	-3.5	-3.8	-5.5	-5.5	-4.1	-2.9	-3.9
Eicosapentaenoicacid	-4	-4	-6.2	-5.1	-4.8	-3.1	-4.9
Erucicacid	-3.3	-4.4	-5.1	-4	-4.2	-2.7	-4.3
EZ-Eicosenoicacid	-2.9	-3	-5.2	-4.3	-3.5	-2.6	-4.1
Gamma-Linolenic acid	-4	-4	-5.6	-4.5	-4.2	-2.2	-4.7
Heptadecanoicacid	-3.9	-3.2	-5.1	-3.8	-3.6	-2.8	-4.5
Lauricacid	-3.8	-3.6	-5.3	-4.5	-3.6	-2.2	-4
Lignocericacid	-3.2	-3.8	-4.8	-5	-3.2	-2.5	-4.1
Linoelaidicacid	-3.8	-4	-4.9	-4.4	-3.3	-3.8	-4.3
Linoleicacid	-4	-3.6	-5.7	-4.1	-4.6	-3.1	-4.9
Linolenicacid	-4.1	-4.1	-5.5	-4.5	-4.2	-2.9	-4.7
Myristicacid	-3.7	-3.5	-5.2	-4.8	-3.7	-2.3	-4.1
Myristoleicacid	-3.4	-3.7	-5.1	-5.1	-4	-2.3	-4.2
Nervonicacid	-3.6	-3.2	-4.8	-4.2	-3.8	-1	-4.5
Oleicacid	-2.8	-3.2	-4.9	-7.6	-4.2	-3	-4.6
Palmiticacid	-3.3	-3.5	-5.4	-4.9	-3.3	-1.4	-4.1
Palmitoleicacid	-3.8	-3	-5.3	-4.8	-4	-3.5	-3.9
Pentadecanoicacid	-3.7	-3.5	-4.9	-4	-3.6	-2.6	-3.8
Stearicacid	-3.6	-3.2	-4.7	-4	-3.5	-2.7	-4.1

The saturated and unsaturated fatty acids derived from natural sources, such as algae, play an important role in the significant biological activity of these organisms. These compounds can act on cell receptors, activating or deactivating biosynthetic pathways and participating in several biological functions (Li et al., 2017), which can often be harmful to other organisms, including parasites (Pineda-Alegria et al., 2020). In this study, the lipid extracts of the two developmental forms of *Iridea cordata*, IFC and IFT, reduced the viability of *T. vaginalis* trophozoites after 24 h of exposure. The IFC lipid extract (600 µg/mL, MIC) reduced viability by 97%, diminishing parasitic development similarly to MTZ ($p > 0.05$), a drug used in the treatment of trichomoniasis.

Even though IFT at MIC (600 µg/mL) caused a trophozoite viability reduction of only 80%, which differs significantly from the values exhibited by MTZ, this value is still considered acceptable when searching for novel antiparasitic molecules. Furthermore, IFT demonstrated a persistent antiparasitic effect, preventing the long-term development of trophozoites and efficiently controlling the growth of *T. vaginalis* trophozoites.

The results indicate a significant difference in the antiparasitic effect of lipid extracts obtained from the same species of macroalgae. This difference can be attributed to the fact that they were obtained from different developmental phases of *I. cordata*. Moreover, in macroalgae extracts the variation of both the presence and quantity of certain substances has been described, and this may be directly affects the pharmacological activity of these extracts, such as the inhibitory action of parasitic development. Such variations have also been reported in a study by Zhao et al. (2017), in which they demonstrated that *Ophiopogon japonicus* cultivated in different geographic regions exhibited differences in their bioactive components. These differences included variations in the concentrations of main components upon extraction, subsequently impacting the pharmacological activity of *O. japonicus* extracts, as stated by the authors.

The antiparasitic effects of natural marine sources have already been reported in the literature, as in the case of lipids isolated from marine sponge species, which showed antiprotozoal activity by inhibiting the amastigote and promastigote forms of *Leishmania* species and targeting the topoisomerase type 1B enzyme, consequently leading to parasite death (Mayer et al., 2021). Furthermore, our study has identified some fatty acids that have previously been associated with potential antiparasitic effects. Atolani et al. (2019) reported that oleic, linoleic, and palmitic acids found in *Polyalthia longifolia* oil were responsible for inhibiting 80.8% of the protozoan *Toxoplasma gondii*. These findings corroborate the results obtained in our study, providing further support for the antiparasitic properties of these fatty acids.

In vitro cytotoxicity testing using the MTT assay was conducted on mammalian VERO and HMVII cells. The results revealed neither extract when tested at its MIC (600 µg/mL), exhibited significant cytotoxic effects after 24 h of incubation. The absence of toxicity observed herein complies with the standards set by the International Organization for Standardization (ISO) in its ISO 10993-5:2009, which was updated in 2017 and describes methods to evaluate the *in vitro* cytotoxicity of medical devices.

These guidelines recommend that cell viability should not be less than 30% when in contact with the evaluated products. Therefore, the results of this study fit this guideline. Furthermore, these values showed no significant difference compared to those exhibited by MTZ, indicating a similar lack of cytotoxicity between the extracts and the synthetic compound currently commercially available for treating *T. vaginalis* infections.

The biological functions of the enzymes PFOR A and B, hydrogenase, and the malic enzyme in the hydrogenosome are mainly related to the energy supply associated with the breakdown of malate and pyruvate, which generates hydrogen and carbon dioxide (Leitsch, 2021). In this study, we investigated the gene expression for these enzymes in *T. vaginalis* trophozoites cultivated in the presence of IFC and IFT extracts, based on which we could assess the changes in the expression levels for these enzymes. The trophozoites exposed to IFC showed a significantly increased expression of hydrogenase and PFOR B, whereas the expression of the malic enzyme was significantly reduced compared to control, DMSO, and MTZ groups.

In the groups treated with IFT, only the expression of the malic enzyme showed a significant increase, with no alterations in the expressions of hydrogenase and PFOR B compared to control groups. Furthermore, both IFT and IFC inhibited the expression of PFOR A when compared to control groups, although the inhibitory actions of both were similar.

PFOR and the malic enzyme catalyze the steps that are essential for the energy metabolism at the *T. vaginalis* hydrogenosome (Rada et al., 2019), whereas hydrogenase acts in the reoxidation of ferredoxin to produce molecular hydrogen, thus, allowing cell growth (Graves et al., 2019). Therefore, we hypothesized that both overexpression and inhibition of these enzymes could have detrimental effects on the parasite. Such effects may include biochemical imbalances that could disrupt metabolic functions that are essential for the survival of this protozoan. This reinforces the antiparasitic activity of IFC and IFT. Furthermore, in this study, we observed that gene expression levels after treatment the IFC and IFT extracts differed from those presented after MTZ treatment. This may suggest a difference in the mechanism of action between the extracts and the commercial drug.

Unlike most protists, *T. vaginalis* is characterized by the presence of hydrogenosomes, a mitochondria-like organelle that is responsible for maintaining the energy metabolism of the parasite (Cheng et al., 2015). Thus, another important target for these compounds are the metabolic pathways in the hydrogenosome of *T. vaginalis*. As the hydrogenosome is metabolically distinct from mitochondria, it becomes an important target for drug development. Certain compounds can selectively act on this organelle to restrict the parasite, which could reduce possible interactions with the host cells. Thus, such substances would be more effective and safer in combating trichomoniasis (Brum Vieira et al., 2015). To investigate the possible mechanisms of action of the IFC and IFT extracts against *T. vaginalis*, we performed an *in silico* analysis with *T. vaginalis* enzymes to verify if they could be modulated could be modulated by the fatty acids constituting both extracts. In this study, the highest docking score (-7.6 kcal/mol) indicated the interaction of oleic acid with the active site of the enzyme methionine

gamma ligase (TvMGL), followed by the interactions between eicosapentaenoic acid and the enzyme lactate dehydrogenase (TvLDH), which had a docking score of -6.2 kcal/mol.

In *T. vaginalis* infections, TvMGL degrades the amino acids that contain sulfur into ammonia, thiols, and α -ketoacids, thus regulating these amino acids by promoting stability in the metabolism of sulfur-containing compounds (Setzer et al., 2017). In contrast, TvLDH acts in the process of glycolysis, carrying out the catalysis of lactate into pyruvate, an interaction that ensures the survival of the parasite. Thus, compounds that can act directly on this enzyme can cause metabolic changes related to the energy production (Neves et al., 2020). As observed in this study, all the compounds evaluated for interaction with the TvLDH binding site showed docking scores that could indicate that interference with this enzyme is associated with the antiparasitic effect of the extracts. Another enzyme that could have its binding site modulated by interactions with compounds present in IFC and IFT is thioredoxin reductase (TvTrxR). TvTrxR showed the highest free energy score of -4.9 kcal/mol when interacting with linoleic acid and eicosapentaenoic acid. Linoleic acid has already been reported to have antiprotozoal action. This enzyme plays an critical role in preventing oxidative damage in *T. vaginalis*. Moreover, TvTrxR is a known target for commercial drugs that disrupt the cellular redox system, causing the death of parasites (Hopper et al., 2016).

Therefore, the docking scores observed in our study characterized important interactions between main compounds from the IFC and IFT extracts and *T. vaginalis* enzymes, indicating they could be potential drug targets. Thus, our data confirmed that these extracts have significant antiparasitic activity against *T. vaginalis* and can be considered promising pharmaceutical products for use in public health.

Conclusion

The lipid extracts from two developmental phases of *Iridea cordata* (IFC and IFT) displayed prominent antiparasitic activity with low levels of cytotoxicity *in vitro*. Furthermore, gene expression analysis indicated that IFC and IFT influenced the expression levels of key enzymes involved in *T. vaginalis* metabolism. This property of these extracts may be responsible for their trichomonacidal action. Additionally, *in silico* analyses demonstrated interactions between the fatty acids in the extracts and proteins vital for *T. vaginalis* survival. In conclusion, this study provides new insights into the potential of natural extracts derived from marine sources as promising options for treating trichomoniasis.

Conflict of interests

The authors declare there are no conflicts of interest.

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