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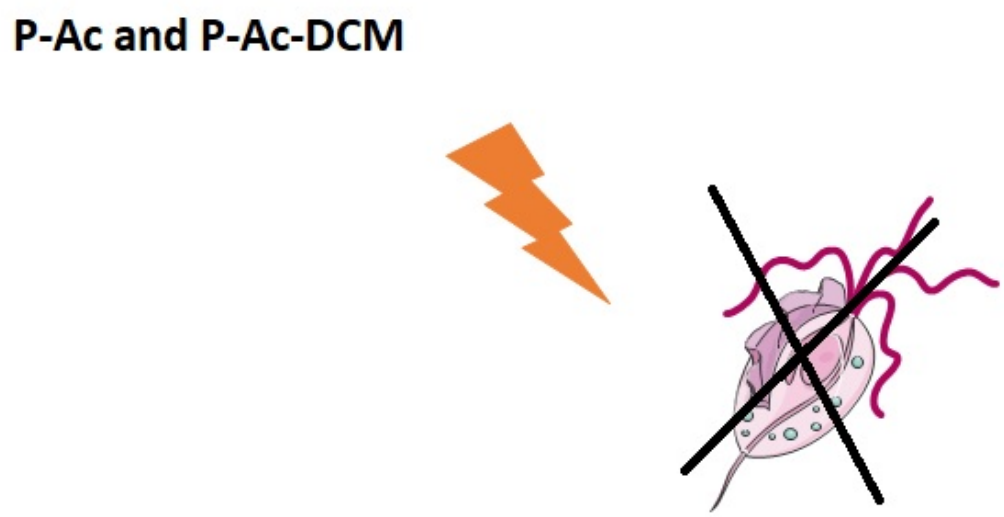

Trichomonas vaginalis is the agent of human trichomoniasis. Both brown propolis fractions, ethyl acetate and ethyl acetate with dichloromethane, were active against this protozoan. The fractions presented no hemolysis and show no cytotoxic effect against VERO and HMVII cell lines.

Anti-*Trichomonas vaginalis* activity of Brazilian brown propolis fractions

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Abstract

Trichomonas vaginalis is the agent of human trichomoniasis, the most prevalent sexually transmitted infection (STI) in the world. The number of asymptomatic cases has increased, which impairs the diagnosis, leads to underestimation of the prevalence and contributes to the classification of neglected disease. Complications due to the infection and failures in treatment motivate the search of new therapeutic alternatives with new mechanisms of action. In this study, the anti-*T. vaginalis* activity of ethyl acetate and ethyl acetate with dichloromethane, the Brazilian brown propolis fractions, was evaluated. The results demonstrated promising anti-*T. vaginalis* activity of both fractions, with MIC values of 500 μg/mL for both fractions and IC_{50} of 83 and 168 μg/mL for ethyl acetate and ethyl acetate with dichloromethane, respectively. The fractions showed low cytotoxicity against the VERO and HMVII cell lines and a low hemolytic effect. These results indicate that the brown propolis fractions present potential anti-*T. vaginalis*; studies on chemical elucidation of the bioactive compound and mechanism of action are in progress.

Keywords: *Trichomonas vaginalis*, brown propolis, ethyl acetate, ethyl acetate with dichloromethane, cell death

1. Introduction

Trichomoniasis is the most common non-viral sexually transmitted infection (STI) in the world, caused by the protozoan *Trichomonas vaginalis*. The World Health Organization (WHO) has estimated an incidence of 276 million new cases per year [1]. In Brazil, the Health Ministry estimates a general prevalence of 15% [2]. *Trichomonas vaginalis* is a parasite which resides in the mucosal of the human urogenital tract, causing vaginitis in women and urethritis in men, although most patients are asymptomatic [3, 4]. This infection has been associated with serious consequences in women, such as adverse pregnancy outcomes, infertility, predisposition to cervical cancer, and pelvic inflammatory disease [5, 6, 7]. The main complications of the disease in men is associated with prostatitis and aggressive prostate cancer [8, 9].

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Although the infection has been considered mild and curable, trichomoniasis is a public health problem. The high prevalence and increasing resistance to the treatment, despite the incentive for protection, including sexual behavior, condom usage, as well as the association with health complications have raised concern to this disease [10]. Infection by *T. vaginalis* has a positive association with both transmission and acquisition of HIV and HPV [11]. Further, the diagnosis, which detects the active motile organism, is still susceptible to failure, and populations of low-income countries lack quick and accurate diagnostic methods [2, 12].

Metronidazole (MTZ) and tinidazole are the only two drugs approved by the FDA (Food and Drug Administration) for the treatment of trichomoniasis and they belong to the same class, the 5-nitroimidazoles [13, 14]. Trichomoniasis treatment with MTZ has shown to produce resistance, allergic reactions, and failure to clear the infection [13]. The mechanism of action is based on the formation of a nitro-radical anion, which is a cytotoxic radical generated by MTZ reduction that inhibits nuclear acid synthesis, leading the parasite to death [14]. Research on new agents for trichomoniasis treatment is urgently required, considering that failures affect an estimated 160000 persons in the United States, and perhaps more than 10 million worldwide that require an alternative treatment [15, 16].

Propolis is a resinous mix produced by bees, which is used to seal and protect the beehive; composition is related to the flora of each region and can be found in the colors green, red and brown [17]. Chemical constituents presented in Brazilian propolis are mainly flavonoids, terpenes, and fatty acids. Brazilian brown propolis is commonly composed of flavonoids, fatty acids, phenylpropanoid acid, and their prenylated derivatives, such as artepillin C [17]. Several activities have already been described for Brazilian brown propolis, such as antimicrobial, antioxidant, anti-inflammatory, anti-*Leishmania* and anti-*Trypanosoma*. The aim of this study was to evaluate the anti-*T. vaginalis* activity of two Brazilian brown propolis fractions.

2. Materials and Methods

2.1. Brazilian brown propolis fractionation

The Brazilian brown propolis sample (100 g) was extracted with ethanol (2.8L) by ultrasonic bath (25 minutes) and processed as described in [16]. The ethanolic extract (1g) was fractionated by accelerated solvent extractor Dionex™ ASE™ 150 using silica gel 230-400 mesh MERCK (39g). The fractions were obtained with dichloromethane (17.8%) and ethyl acetate (44.2%). The parameters used in the extractor were the following: temperature of 100 °C, static extraction cycle of 1 minute, 150% volume wash, pressure of 1,500 psi and purge of 100 seconds. All the fractions were concentrated on rotary evaporator and freeze-dried. Two Brazilian brown propolis fractions were obtained and tested in the following assays: ethyl acetate (P-Ac) and ethyl acetate with dichloromethane (P-Ac-DCM).

2.2. Culture in vitro of T. vaginalis

The isolate *T. vaginalis* ATCC30236 was cultured *in vitro* in trypticase-yeast extract-maltose (TYM) medium, pH 6.0, supplemented with 10% (v/v) heat inactivated serum, and incubated at 37 °C [18]. Parasites in the logarithmic phase of growth and normal morphology were used for furthers assays.

2.3. Minimum inhibitory concentration (MIC)

MIC value, which is the minimum concentration able to kill 100% of parasites, was determined in 96-well microtiter plates. The fractions P-Ac and P-Ac-DCM were added in TYM medium to obtain a serial dilution (500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 μg/mL). Subsequently, trophozoites suspension was added at a 2.0×10^5 trophozoites/mL final density. Two negative controls were prepared: parasites only (negative control) and vehicle control for solubilization of the derivatives (0.6% DMSO). After 24 hours of incubation at 37 °C, 5% CO_2, the parasites were counted in hemocytometer using Trypan blue dye exclusion (0.2% v/v) and analyzed for their motility and morphology. The wells corresponding to MIC value and concentrations below, as well as controls, were inoculated in fresh TYM medium at 37 °C. The parasites were analyzed every 24 hours for 120 hours to confirm MIC.

2.4. Hemolytic assay

The rate of hemolysis was performed as described by [19], with some modifications [20]. The Federal University of Rio Grande do Sul Research Ethical Committee approved documents, procedures, and project under authorization CAAE 47423415.5.0000.5347. Erythrocytes were obtained from the heparinized blood of healthy human donors. They were washed three times with PBS 1x (pH 7.0; 37 °C) at 3000 RPM for 5 minutes and resuspended to obtain a 5.0×10^7 cells/mL. Then, erythrocytes suspensions were incubated with eightfold serial dilution of each fraction for 24 hours at 37 °C. After, hemoglobin released was measured at 540 nm. Two controls were prepared: a negative control (erythrocytes and PBS 1x) and a positive control (erythrocytes and Triton X-100 0.2%). Results were expressed as the hemolysis percentage of each compound, comparing to the 100% hemolysis that was attributed to the hemolytic action of the positive control 0.2% Triton X-100.

2.5. Kinetic growth assay

The *T. vaginalis* ATCC 30236 isolate at density of 2.0×10^5 trophozoites/mL was incubated in TYM medium with the fractions at MIC values. Counting of viable trophozoites with hemocytometer was performed at 2, 4, 6, 12, 24, 48, 72, 96, and 120 hours. Parasites in fresh TYM medium as negative control and in 0.6% DMSO as vehicle control were included along the assay, and submitted to the same evaluation. Results were expressed as trophozoites/mL by comparing treated via viable trophozoites with untreated parasites.

2.6. Cytotoxicity against mammalian cells

The HMVII (vaginal epithelial cells) and VERO (kidney epithelial cells from African green monkey) cell lineages were cultured in RPMI and Dulbecco's Modified Eagle's Medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C, 5% CO_2. For the test, 1.0×10^5 cells/well were seeded in 96-well microtiter plates for 24 hours. After, medium was replaced with fresh medium containing eightfold serial dilution of each fraction. Three controls were prepared: negative control with no compound, vehicle control (DMSO 0.6%) and positive control (Triton X-100 0.2%). The plates were incubated for 48 hours. After, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) was added and incubated for one hour at 37 °C. MTT was removed and the insoluble purple formazan product was dissolved in DMSO. The amount of reduced MTT was measured at 570 nm. Results were expressed as percentage of viable cells compared to negative control.

2.7. Statistical analysis

All experiments were performed in triplicate and with at least three independent cultures (n = 3). Data were expressed by mean ± standard deviation (S.D.). Statistical analysis was conducted using the Student's *t*-test and a 5% level of significance was applied to the data. The software GraphPad Prism (San Diego, CA) was used for IC_{50}, CC_{50} and HC_{50} determination by non-linear regression and used to determine the selectivity index (SI), where SI is $\frac{CC_{50} \text{ or } HC_{50}}{IC_{50}}$.

3. Results

3.1. Brazilian brown propolis fractions presented anti-T. vaginalis activity

In order to evaluate the anti-*T. vaginalis* activity of Brazilian brown propolis, MIC and IC_{50} values were determined. The concentrations of both fractions revealed potential anti-*T. vaginalis* activity with MIC and IC_{50} values of 500 and 83 μg/mL for P-Ac fraction, as well as 500 and 168 μg/mL for P-Ac-DCM fraction, respectively. The results can be seen in Table 1. The effects of these extracts at MIC and IC_{50} were investigated in the subsequent experiments.

Table 1: Anti-*Trichomonas vaginalis* activity, cytotoxicity against VERO and HMVII lineages and hemolysis of fractions from Brazilian brown propolis.

Fraction		P-Ac	P-Ac-DCM
ATCC30236	MIC	500 μg/mL	500 μg/mL
	IC_{50}	83 μg/mL	168 μg/mL
HMVII	CC_{50}	224 μg/mL	250 μg/mL
	SI	2.69	1.48
VERO	CC_{50}	261 μg/mL	331 μg/mL
	SI	3.14	2.01
Hemolysis	HC_{50}	225 μg/mL	150 μg/mL
	SI	2.70	0.89

3.2. Brazilian brown propolis fractions presented low hemolytic effect

Hemolysis assay demonstrates the compatibility of compounds against erythrocytes. Table 1 shows HC_{50} values of 224 μg/mL and 150 μg/mL for P-Ac and P-Ac-DCM fractions, respectively. SI values were determined as 2.70 and 0.89 for P-Ac and P-Ac-DCM fractions, respectively.

3.3. Brazilian brown propolis fractions presented low cytotoxicity

In order to evaluate the safety against mammalian cells, MTT assay were performed and used to determinate the selectivity of the P-Ac and P-Ac-DCM fractions. Table 1 shows the results of HC_{50} for VERO and HMVII cell lineages. The positive control Triton X-100, as expected, reduced the cell viability whereas the vehicle control caused no damage. The P-Ac fraction showed low cytotoxicity for both VERO and HMVII, with SI values 3.14 and 2.69 respectively. The P-Ac-DCM fraction showed SI values 2.01 for VERO and 1.48 considering HMVII cells.

3.4. Brazilian brown propolis fractions inhibited T. vaginalis growth

To analyze the effect of P-Ac and P-Ac-DCM on *T. vaginalis* proliferation, kinetic growth experiments were performed. An initial inoculum of 2.0×10^5 trophozoites/mL was incubated in the presence of both fractions at MIC values. The results show the expected curve of viable control trichomonads, as demonstrated in Figure 1. In contrast, fraction treated parasites presented a significant reduction in growth at 6 hours for P-Ac fraction and at 4 hours for P-Ac-DCM fraction. The trichomonads growth was abolished by both fractions after 24 hours of incubation.

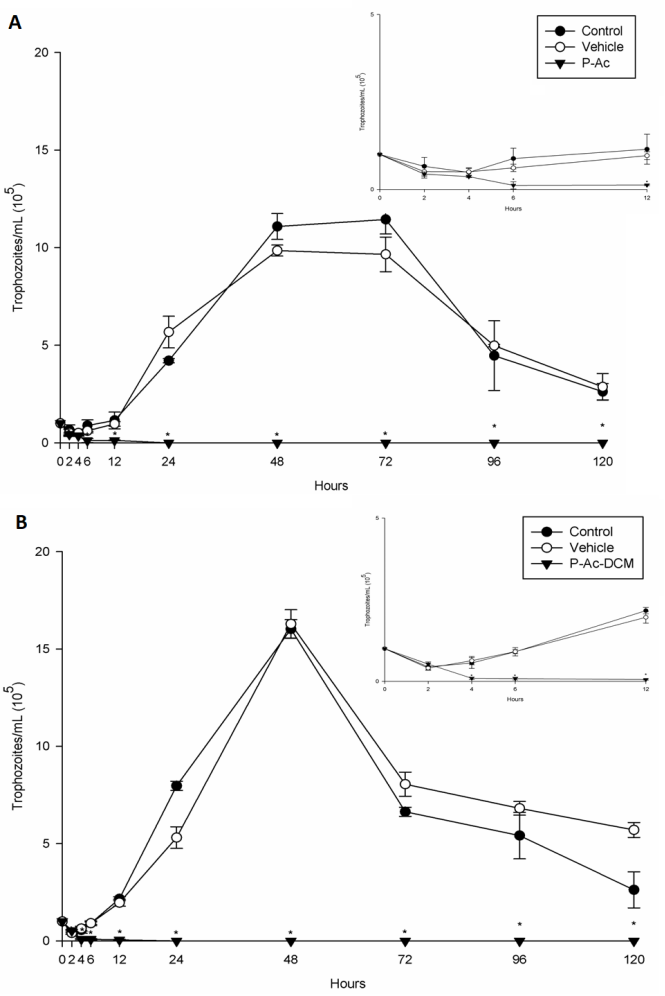

Figure 1: Effect of ethyl acetate (P-Ac) and ethyl acetate with dichloromethane (P-Ac-DCM) fractions of brown propolis in *T. vaginalis* kinetic growth at MIC value, in comparison to untreated parasites (control) and parasites with 0.6% DMSO (vehicle). Data represent mean ± standard deviation of three experiments in triplicate. (*) Statistically significant difference ($p < 0.05$) when compared to the negative control by the Student's *t*-test.

Table 2: Review in literature (PubMed, 2006-2017) on biological activities described for Brazilian red, green and brown propolis.

Propolis	Microorganism	MIC	References
Red Propolis	Antibacterial		
	Enterococcus faecalis	nd*	[21, 22, 23]
	Escherichia coli	nd*	[24, 23]
	Neisseria meningitidis	nd*	[23]
	Staphylococcus aureus	25 to 50 μg/ml 62.5–125μg/mL 3.8 μg/mL 200-400μg/mL 31.2 to 125 μg/mL nd*	[25] [26] [21] [27, 28] [24] [23, 29]
	Streptococcus mutans	62.5–125μg/mL 28 μg/ml 25 to 50 μg/ml 31.2 to 62.5μg/mL nd*	[26] [27] [28] [25] [23, 21]
	Antifungal		
	Candida albicans, C. glabrata, C. krusei, C. parapsilosis	nd*	[24, 30, 23]
	C. glabrata *C. parapsilosis*	3.9μg/mL 15.62 μg/mL	[31]
	Saccharomyces cerevisiae	nd*	[32]
	Trichophyton spp.	1024 μg/mL	[33]
Green Propolis	Antibacterial		
	Bacillus subtilis	125 μg/mL	[34]
	Bifidobacterium spp., *Neisseria* spp., *Streptococcus acidominimus*, *Streptococcus oralis*, *Staphylococcus epidermidis*, *Veillonella parvula*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus acidophilus*	nd*	[35]
	Escherichia coli	256 to 512 μg/mL	[36]
	Helicobacter pylori	0.075 mg/mL	[37]
	Staphylococcus aureus	50 to 100 μg/mL 500 μg/mL nd*	[36] [34] [38]
	Streptococcus mutans	2.08 mg/mL 500 μg/mL	[39] [40]
	anti-*Trypanosoma*		
	Trypanosoma cruzi	IC_{50} 8.5 μg/mL	[41]
	anti-*Leishmania*		
	Leishmania (Viannia) braziliensis	nd*	[42]
	Antifungal		
	Candida spp.	nd*	[43, 44]
Brown Propolis	Antibacterial		
	Bacillus subtilis	31.25 μg/mL	[34]
	Enterococcus faecalis	500μg/mL 860 μg/mL 1000 mg/mL	[45] [36] [46]
	Escherichia coli	13.9 μg/mL nd*	[36] [47]
	Staphylococcus aureus	19.9 μg/mL 15.62 μg/mL	[36] [34]
	anti-*Trypanosoma*		
	Trypanosoma cruzi	nd*	[48]
	anti-*Leishmania*		
	Leishmania amazonensis	nd*	[49]
	Antifungal		
	Sporothrix brasiliensis *Cryptococcus neoformans*	0.19 to 1.56 mg/mL	[50]

4. Discussion

Despite being the most common non-viral STI in the world, presenting incidence levels higher than chlamydia, gonorrhea, and syphilis infections combined, trichomoniasis is still considered as neglected [10]. Research for new compounds, either synthetic or natural, is motivated by increase in resistance. Currently, it can be estimated that 10% of cases worldwide presented some level of resistance, making difficult the elimination of the parasite and exposing the patient to high levels of MTZ or tinidazole treatment and, consequently, to drug adverse effect.

Propolis has been used in traditional medicine to treat different diseases [51], and presents antioxidant capacity, antibacterial, antibiofilm. Red and green Brazilian propolis have shown antibacterial activities [52]. Recently, [17] has demonstrated a new potential application for Brazilian brown propolis, with activity against *Candida albicans*; however, only Cuban propolis extracts was evaluated against *T. vaginalis* [53]. Table 2 summarizes the different studies in the literature on the effects of propolis. MIC values marked as "nd" were not given by the authors. It has been shown to have antibacterial, antifungal, anti-*Leishmania* and anti-*Trypanosoma* activities, besides anti-inflammatory, anti-caries, antioxidant, antitumor and even ability to activate the immune response. According to the type of propolis, MIC and IC_{50} values may vary from 0.19 to 1000 μg/mL, revealing the potential of propolis.

The present study tested the fractions P-Ac and P-Ac-DCM of brown propolis for anti-*T. vaginalis* activity. Both fractions presented MIC values of 500 μg/mL, and IC_{50} values of 83 and 168 μg/mL, respectively. In addition, to better understand the inhibition profile of the fractions, a kinetic growth curve experiment has been conducted where the trophozoites were incubated with the fractions for 120 hours, at MIC values. Both fractions showed a significant difference in proliferation, 6 hours for P-Ac and 4 hours for P-Ac-DCM. After 24 hours of incubation, both fractions complete inhibited the parasite proliferation. These results corroborate that both fractions show potential for anti-*T. vaginalis* activity and can be used to further investigations about parasite death mechanisms.

In order to investigate the safety of fractions, cytotoxicity against mammalian cell lines and erythrocytes was performed for the determination of parasite selectivity. The results indicate that both fractions are slightly hemolytic after incubation with erythrocyte suspension, presenting SI of 2.7 when treated with ethyl acetate fraction. Subsequently, the cytotoxic effect was evaluated in VERO and HMVII lineage through the incubation with the extracts. VERO and HMVII have the same behavior, they showed a slight reduction in the cellular viability. Cytotoxicity evaluation allows determinate the selectivity of compounds, which suggest if the compound is targeting more the pathogen then the cellular host. Thus, an SI cut-off equal or greater than 1.0 means that the fraction caused less damage to the host then to parasite. P-Ac showed SI of 3.14 and 2.69 of VERO and HMVII, respectively. While P-Ac-DCM showed SI of 2.01 and 1.48 for VERO and HMVII. These results highlight the greater tolerance and safety of brown propolis extracts against host cells.

Natural compounds make up about 35% of approved drugs or semisynthetic derivatives, while 30% are synthetic molecules inspired by natural products or present a pharmacophore developed from natural compounds [54]. Ancient medicine uses plants for the treatment of various disorders. Propolis has been used in traditional medicine since ancient times to treat different diseases and has been used as anti-inflammatory and antitumor. Natural products provide an immeasurable wealth of active molecules and facilitate the development of new therapies, since they are typically easier to obtain than artificial compounds. The structural diversity may result in distinct mechanisms of action. In this sense, these data corroborate the use of Brazilian propolis

as a source of active molecules for the development of alternative therapy against trichomoniasis and the increasing current resistance.

5. Conclusion

Sexually-transmitted illnesses are public health problem and cause relevant complications. Trichomoniasis, a neglected parasitic infection, presents high transmission and consequently, increases HIV/AIDS acquisition. Studies of new compounds such as the brown propolis fractions are needed to search new alternatives to combat this pathogen with new mechanisms, since the current drugs present failures in the conventional treatment. Further studies are necessary to prove the potential of the Brazilian brown propolis as anti-trichomonal agent.

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