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Properties of constituents from *Maytenus gonoclada* against *Entamoeba histolytica* and two leukemia cell strains

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Species of genus *Maytenus* represents promising sources of bioactive compounds, mainly pentacyclic triterpenes of medicinal interest. The properties of crude extracts and three known triterpenes obtained from branches and roots of *Maytenus gonoclada*, against *Entamoeba histolytica* and human promyelocytic leukemia HL-60 (myeloid leukemia), and Jurkat (lymphocytic leukemia) cells were *in vitro* evaluated. For crude extracts, lupeol and 3-oxo-11α-hydroxylup-20(29)-ene activity were not observed against *E. histolytica*, and neither against leukemic cells. Nevertheless, in relation to tingenone considerable biological effect was detected. The cytotoxic effect of tingenone was evaluated upon human peripheral blood mononuclear cells. The cytotoxic dose showed higher efficacy, compared to the other assays. The present study signalizes the importance of tingenone, as well as its use in the production of derivatives with potential antiparasitic and antitumoral properties.

Key words: *Maytenus gonoclada*, anti-*Entamoeba histolytica* activity, anti-leukemic cells activity.

INTRODUCTION

Among the tropical diseases, amoebiasis represents the second most frequent cause of death associated to

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parasitic infections, especially in developing countries. High prevalence of *Entamoeba histolytica* is observed mainly in Mexico, Central and South America, including Brazil, Africa, India and other tropical regions (Tanyuksel and Petri, 2013). About 500 million people worldwide are annually infected with *E. histolytica* (Herbinger et al., 2011), which is responsible for 50 million clinical cases of dysentery or amoebic liver abscess, culminating in about 100.000 deaths (Tanyuksel and Petri, 2013).

The parasite commonly lives in large intestine, causing changes in gastrointestinal mucosa, which characterize the clinical form of the disease called intestinal amoebiasis. In some persons, the *E. histolytica* invades the intestinal submucosa reaching bloodstream, and then target organs such as liver, lung, heart, skin and others. The incidence of histological changes characterizes the profile of extra-intestinal amoebiasis (Ximénez et al., 2011).

The metronidazole (MTZ) is the principal prescribed drug used in the treatment of amoebiasis. Short-term exposure, exposition to toxic levels of MTZ, such as in prescriptions aiming prophylactic, and noncompliance treatments, frequently represent the processes that establishes conditions under which the drug resistance is induced (Pritt et al, 2008; Upcroft and Upcroft, 2001). The main reason for noncompliance to treatment is the collateral symptoms of MTZ, such as nausea, metallic taste, headache, dizziness, insomnia beyond other undesirable effects (Cudmore et al., 2004).

By means of ethnopharmacological studies, it has been shown that substances obtained from plants could present therapeutic activity associated to some diseases. For this reason, in the last decades a great variety of vegetable species used in traditional medicine have been investigated to evaluate and validate its real pharmacological properties. In development countries, medicinal plants have been traditionally used to treat gastrointestinal diseases including those induced by parasites. Within this context, the species *Autroplenckia populnea* Reissek (Celastraceae) has being considered of great interest for researchers in function of its properties, such as anti-diarrheal, antitumoral and antirheumatic, etc (Miranda et al., 2009). Species of *Maytenus*, another genus of the Celastraceae family, also have been used in traditional medicine of Brazil and other countries to treat a variety of illnesses, including diarrhea (Niero et al., 2011). As example, crude extracts of *Maytenus imbricata* showed an expressive inhibitory activity against strains of *Trichomonas vaginalis*, an intestinal parasite that is transmitted by the fecal-oral route and that present strains sensitive and resistant to MTZ (Batista et al., 2014).

M. gonoclada, is a native species, commonly found in “Cerrado” (Savanna region) of Brazil (Oliveira-Filho and Machado, 1993). The typical climate of "Cerrado" regions is hot, semi-humid and seasonal, characterized by rainy summers and dry winters. In general, the soil of this region is deep, very old and chemically poor in terms of nutrients. Due to these environmental conditions, plants that grow in “Cerrado” regions exhibit quite specific biological and chemical characteristics. By means of phytochemical methods applied to different extracts from leaves and branches of *M. gonoclada*, until this moment were isolated and identified different pentacyclic triterpenes, mainly those of the friedelane and lupane series (Oliveira et al., 2007; Silva et al., 2011). Pentacyclic triterpenes represent a big class of natural compounds that are formed by sequential cyclization of squalene, and the different types of skeleton formed, transforms these compounds into a promising group of secondary metabolites (Laszczyk, 2009). These triterpenes are considered as important structural constituents involved in the stability of phospholipid bilayers of plant cell membrane, just as cholesterol is in animal cells (Saleem, 2009). Several biological activities have been attributed to pentacyclic triterpenes (Silva et al., 2011). The 3β,6β,16β-trihydroxylup-20(29)-ene, a member of lupane series, has potent inhibitory activity against promastigotes of *Leishmania amazonensis* (Teles et al., 2011). Among quinonamethides, other class of pentacyclic triterpenes, tingenone and pristimerin, which until this moment only were isolated from roots of some Celastraceae species, showed *in vitro* activity against *Trypanosoma cruzi* and *Plasmodium falciparum* (Kayser et al., 2002). The structural diversity of pentacyclic triterpenes that possess potential antiprotozoal property, involved in different mechanisms of action, has stimulated the interest in the identification of other natural compounds that may provide new antiparasitic drugs.

In this work, crude extracts from branches and roots and the triterpenes 3-hydroxy-24,29- dinor-1(10),3,5,7-friedelatetraen-2,21-dione (tingenone) (compound 1), 3-oxo-11α-hydroxylup-20(29)-en (compound 2) and 3β-hydroxylup- 20(29)-en (lupeol) (compound 3) (Figure 1), both isolated from non-polar extracts of *M. gonoclada*, were subjected to assays aiming to evaluate its activity against the protozoan *E. histolytica*.

Antitumoral properties were previously attributed to some pentacyclic triterpenes (Laszczyk, 2009; Saleem, 2009), among them is tingenone which showed activity against adenocarcinomas murines of lung (LP07) and adenocarcinomas murines of breast (LM3) (Gomes et al., 2011). Due to this fact, the cytotoxic effect of compounds 1, 2 and 3 was established *in vitro* using cell culture of human promyelocytic leukemia HL-60 (myeloid leukemia) and Jurkat (lymphocytic leukemia). The safety of these compounds was evaluated on culture of human peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

General experimental procedures

Glass tube (25 mm × 65 cm) filled with silica gel 60 (70–230 Mesh,

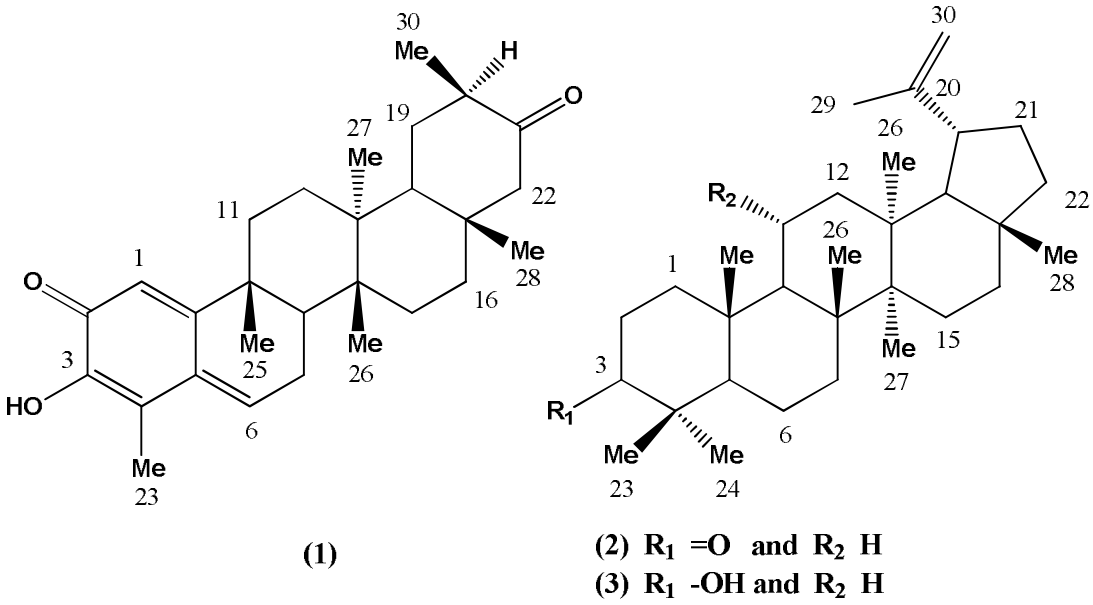

Figure 1. Chemical structure of 3-hydroxy-24,29- dinor-1(10),3,5,7- friedelatetraen-2,21-dione (tingenone) (compound 1), 3-oxo-11α-hydroxylup-20(29)-en (compound 2) and 3β-hydroxylup- 20(29)-en (lupeol) (compound 3) isolated from *Maytenus gonoclada*.

erck) was used for column chromatography (CC) and precoated silica gel plates (Merck) for thin layer chromatography (TLC). The detections of spots were made by spraying a mixture (1:1) of vanillin (ethanol solution 1% w/v) and perchloric acid (aqueous solution 3% v/v). A Mettler FP 80 HT apparatus was used to determine melting points (uncorrected). Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-IR) was performed on a Perkin Elmer, Spectrum One spectrophotometer.

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured on a Bruker DRX 400 Avance spectrometer at 400 MHz at 300 K, equipped with inverse detection 5 mm multinuclear head $^{1}H/^{13}C$. Each compound was dissolved in $CDCl_3$ and transferred to a 5 mm OD NMR tube. Tetramethylsilane (TMS) was used as internal standard ($\delta_H = \delta_C = 0$). Bi-dimensional (2D) NMR spectra were acquired under standard conditions. Data processing was carried out on SGI workstation using the Bruker (DRX 400) software.

Plant

Branches of *Maytenus gonoclada* Martius were collected in 2004, in Serra da Piedade, Caeté, Minas Gerais, Brazil and the roots was collected in 2010. An exsiccata, of the plant collected and identified was deposited (n° 60280) at the Herbarium of the Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

Extracts preparation

The branches and roots of *M. gonoclada* were dried over Kraft paper at room temperature. The roots were separated into bark and wood. Each part of the dried plant was ground using a knife mill. The powdered material obtained from branches was exhaustive and sequentially extracted in a Soxhlet apparatus, with hexane, chloroform, ethyl acetate and ethanol. The bark and wood obtained from roots were respectively subjected to exhaustive extraction in a Soxhlet apparatus, with hexane/ethyl ether (1:1), chloroform, ethyl acetate and finally with ethanol. Each extract solution was concentrated under vacuum in a rotary evaporator at temperature below 45°C.

Isolation of triterpenes

The chloroform extract from branches and the hexane/ethyl ether (1:1) extract from root bark gave test of Liebermann-Burchard positive for pentacyclic triterpenes (Ghosh et al., 1985). Subsequently, these extracts were subjected to column chromatography procedures. Triterpene (compound 3) (10.0 mg) was isolated from chloroform extract (5.0 g) of branches through silica gel CC eluted with hexane/chloroform/ethyl acetate (7:2:1) (Silva et al., 2011). The extract of the bark (500 mg) obtained using hexane/ethyl ether (1:1) was submitted to silica gel CC eluted in gradient polarity conditions, yielding constituent compound 2 (17.7 mg) (fraction hexane/ethyl acetate 8.8:1.2) and compound 1 (20.5 mg) (fraction hexane/ethyl acetate 8.5:1.5). The chemical structure of triterpenes (compounds 1, 2 and 3) was respectively confirmed by means of the respective melting point, ATR-IR, ^{1}H and ^{13}C NMR spectral data, and also by comparison with authentic sample through TLC.

Culture and growth conditions

E. histolytica (EGG) strain was isolated and axenized in Brazil, from male patient with dysenteric colitis and liver abscess. This strain was used in all experiments, being kept in a casein-free medium, YI-S, consisting of a nutrient broth, vitamin mixture and serum (Diamond et al., 1995) and maintained incubated at 37°C. To quantify the effect of triterpenes (compounds **1**, **2** and **3)**, trophozoites (2.4×10^5) were grown in glass culture tubes (Pyrex®) for 48 h.

Preliminary screening

Each extract and triterpenes (compounds 1, 2 and 3) were

previously dissolved in 1.0 ml of dimethylsulfoxide (DMSO), and an aliquot of the solution was added to 10 ml of YI-S culture medium. These solutions were sterilized using a nitrocellulose membrane filter (0.22 µm) and added to trophozoite glass culture tubes to achieve final concentration test of 17.0 µg/ml (crude extracts) and 100.0 µM (triterpenes 1, 2 and 3). The active compounds were evaluated subsequently to determine the inhibitory concentration that reduces 50% trophozoites growth (IC_{50}).

Amoebicidal assay

The active substances and MTZ were respectively dissolved in DMSO (1.0 ml), reaching concentrations from 14.0 to 20.0 mg/ml. A sample (200.0 µl) of each solution was added to 10 ml of YI-S culture medium to obtain final concentration ranging from 0.28 to 0.40 mg/ml. The solutions were sterilized using a nitrocellulose membrane filter (0.22 µm). Aliquots of each solution were added to trophozoite glass culture tubes, separately, in increasing concentrations test of 1.0 to 32.0 µM for triterpenes 1, 2 and 3, and of 0.4 to 12.8 µM for MTZ. After 48 h incubation at 37°C, the viability of cells was qualitatively established observing the mobility and adhesion of trophozoites using an Olympus inverted microscope (IX51). The culture tubes were placed on ice bath to detach the trophozoites. After 20 min each, culture tubes were shaken with the vortex mixer at the low mixing position to get trophozoites suspension to be counted in Neubauer's Chamber. The viability of trophozoites was determined by eosin exclusion (Carvalho and Silva, 1998). The effect of substance was evaluated through the comparison with the negative control (inoculum and culture medium) and a positive control (MTZ). All assays were done in triplicate and repeated twice. Data on the percent inhibition for each concentration of test substance were compared by analysis of variance (ANOVA) using Minitab 15 statistical software. The IC_{50} values were graphically obtained from dose-effect curves using Prism 5.0 (GraphPad Software Inc.).

Human PBMC cells cytotoxicity assay

The protocol previously described (Souza-Fagundes et al., 2003), had some modifications as follows. PBMC were isolated from heparinized venous blood of healthy both sexes adult volunteers. Samples of PBMC were obtained through agreement with Fundação Centro de Hematologia e Hemoterapia de Minas Gerais (Hemominas), Brazil (Protocol no 105/2004). The heparinized blood was placed in glass culture tubes containing Ficol/Histopaque®. After centrifugation (1400 rpm) during 40 min at 18°C, the PBMC were collected from the interphase. The cells were washed with RPMI-1640 formulation (Sigma Aldrich). The cell suspension was adjusted to 2.0×10^6 cells/ml and 100 µl added to each well. The cells were cultivated in complete RPMI-1640 in flat-bottomed microtiter plates (Costar, tissue culture treated polystyrene). PBMC culture growth was stimulated with phytohemagglutinin (PHA) 25 µg/ml and incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO_2 in the presence or absence of test compounds. Active substances and MTZ were dissolved, separately, in DMSO prior to dilution. The IC_{50} was determined over a range of concentrations (100 nM to 100 µM). All cultures were maintained at 37°C in a humidified incubator with 5% CO_2 for 48 h. For comparison, the cytotoxicity of cisplatin was evaluated under the same experimental conditions. All cultures were carried out in RPMI-1640 medium (Sigma-Aldrich), supplemented with 5% (v/v) human serum, type AB, heat inactivated (GIBCO) and 2 mM L-glutamine. The antibiotic/antimicotic solution (1000 U/ml penicillin, 1000 µg/ml streptomycin and 25 µg/ml fungisone) (GIBCO) was added to prevent fungal and bacterial contamination. Cell proliferation and viability were determined using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (2.5 mg/ml) (Monks et al., 1991). Optical density (OD) measurements at 590 nm were taken on a Perkin Elmer UV/Vis Spectrometer Lambda model. Results were normalized with DMSO control (0.05%) and expressed as a percentage of cell viability inhibition. Interactions of compounds and media were estimated on the basis of the variations between the drug-containing medium and drug-free medium to control for false-positive or false-negative results. The IC_{50} values were graphically obtained from dose-effect curves using Prism 5.0 (GraphPad Software Inc.). The cytotoxicity of cisplatin was evaluated under the same experimental conditions. The experiments were performed using blood of eleven healthy both sex donors and all treatments were performed in triplicate.

HL-60 and Jurkat cells assays

The lineages of human promyelocytic leukemia HL-60 (myeloid leukemia), and Jurkat (lymphocytic leukemia) cells were cultivated to log phase in RPMI1640 (Sigma-Aldrich), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO), and enriched with 2 mM of L-glutamine and 10% fetal bovine serum (FBS). The cultures were maintained at 37°C in a humidified incubator with 5% CO_2.

Cell suspensions of HL-60 and Jurkat cells were seeded on 96 wells microtiter plates at a concentration of 5×10^4 and 105×10^4 cell/well, respectively, and incubated for 6 h at 37°C for culture stabilization. A stock solution (20.0 mM/ml) was prepared dissolving triterpenes 1, 2 and 3 in DMSO. Subsequently, each triterpene solution was added to leukemia cell cultures through serial dilutions ranging from 10 nM to 100 µM, and incubated at 37°C for 48 h, in a humidified atmosphere of 5% CO_2. The proliferation and cell viability were assessed by MTT method at 590 nm (Monks et al., 1991). Results were normalized with DMSO-treated (0.5%) control cell and expressed as a percentage of cell viability inhibition. Interactions of compounds and media were estimated on the basis of the variations between the drug-containing medium and drug-free medium to control for false-positive or false-negative results. The procedure, in triplicate, was performed in two independent experiments, using cisplatin as positive control. The IC_{50} values were graphically obtained from dose-effect curves using Prism 5.0 (GraphPad Software Inc.).

RESULTS AND DISCUSSION

Considering that antiprotozoal properties of *Maytenus* genus has previously been associated with the triterpenoids (Silva et al., 2011), preliminary assays were conducted in order to verify possible effects induced by crude extracts and constituents 1, 2 and 3 obtained from *M. gonoclada* on the growth and viability of trophozoites of *E. histolytica*. In the present work, it was observed that crude extracts from branches and roots (bark and wood) showed no activity against *E. histolytica* (data not shown). Considering the complexity of substances usually present in crude extracts (Barreto Júnior et al., 2005), it is possible to suggest that constituents interacts among them causing inhibition, competition or physical impediment of the target of action of the active substances.

The antiprotozoal properties of some species of the *Maytenus* genus (Santos et al., 2013) can be associated with the triterpenoids. As described in the present study,

Table 1. IC_{50} of metronidazole (MTZ) and triterpene 1 (tingenone) after 48h incubation time.

Compounds	IC_{50} (µM)	Confidence interval (95%)
MTZ	1.26	(0.88 – 1.80)
Triterpene 1	4.08	(2.95 – 5.63)

Table 2. Median inhibitory concentration (IC_{50}) of triterpene **1** determined *In vitro* against PBMC, HL-60 and Jurkat cells lines

Cell culture	IC_{50} (µM)[a] of compound	
	Triterpene 1	Cisplatine[c]
PBMC	8.9 (6.5 - 12.1)[b]	3.4 (2.8 - 5.9)[b]
HL-60	2.1 (1.4 - 2.3)[b]	0.005 (0.003 - 0.22)[b]
Jurkat	1.1 (0.4 - 3.1)[b]	17.54 (8.9 - 34.5)[b]

[a]Concentration that inhibit 50% cell growth. [b]95 % confidence interval ($P < 0.05$). [c]Anticancer drug used as referential

the pentacyclic triterpenes represents the principal constituents of *M. gonoclada* (Silva et al., 2011), mainly in the crude hexane/ethyl ether (1:1) extract from its root bark. Three triterpenes (1, 2 and 3) were isolated, chemically identified and subjected to assays aiming to observe its activity against *E. histolytica*. Among constituents 1, 2 and 3, only compound 1 (tingenone) showed activity, in preliminary screening. From the results, it is possible to conclude that compound 1 have amoebicidal property. Unlike the other triterpenes isolated compounds (2 and 3), only compound 1 is soluble in water. This property facilitated the diffusion of compound 1 in the culture medium, generating good conditions to reach the highest activity. The polarity of the substances is important for its antimicrobial activity, due to the diffusion in biological medium (Araújo et al., 2009). The low polarity of triterpenes 2 and 3 suggested that the lack of activity against *E. histolytica* may be related to its insolubility in the culture medium. The mechanism of tingenone (compound 1) action is not well elucidated. In accordance to literature, the molecular mechanism suggest a possible mode of action involving quasi-intercalative interaction of the compounds with DNA, followed by nucleophilic interaction of the DNA base and the carbon C-6 of this triterpene (Zandi et al., 2010). Based on the results, triterpene 1 and the MTZ were subsequently evaluated at different concentrations, to determine their IC_{50}. After incubation period with *E. histolytica* culture, both compound 1 and MTZ induced an increase of the size and vacuolization, loss of mobility, reduced adhesive capacity and death of trophozoites. Comparing the IC_{50} values found, triterpene 1 (4.08 µM) was less active than MTZ (1.26 µM) (Table 1).

Nevertheless, triterpene 1 is the main metabolite found in *M. gonoclada* and *M. imbricata*, from which large amounts are easily isolated (Veloso et al., 2014), making feasible their use. In addition, tingenone (compound 1) was previously evaluated against *Giardia lamblia* showing an IC_{50} similar to MTZ (Mena-Rejón et al., 2004) proven to be strong modulator of the immune system (Moreira et al., 2001) and further, a total growth inhibition of *T. cruzi* (Godjiman et al., 1985) suggesting the potential of this quinonamethide triterpene as a model for news antiparasitic agents.

In a previous report the antitumoral property of tingenone was described (Reyes et al., 2011). Due to this fact, in this present work, compound 1 was assayed against cell strains of human promyelocytic leukemia HL-60 (myeloid leukemia), and Jurkat (lymphocytic leukemia). Jurkat cells have not yet been sufficiently evaluated as target for antileukemical drugs. The antitumoral activity of copound 1 was tested on tumor cell lines, HL-60 and Jurkat and the IC_{50} found was 2.1 µM and 1.1 µM, respectively (Table 2).

The cytotoxic effect of compound 1 was evaluated upon human PBMC cells. The cytotoxic dose was higher than the effective one for all assays. The cytotoxicity of MTZ and compound 1 were respectively evaluated on PBMC. MTZ showed no significant inhibitory effect at concentrations below 100.0 µM. The IC_{50} found for compound 1 was 8.9 µM, similar cytotoxicity against human PBMC when compared with cisplatin, used as positive control (Table 2).

As compared to cisplatin, a drug used in anti-cancer therapy (Cozzolino et al., 2005), antileukemia activity of triterpene 1 related to Jurkat cells was considered up to about 16 times higher and 2.6 times less cytotoxic. These results are in agreement with Reyes et al. (2011) who affirm that tingenone shows cytotoxic activity against cancer cell lines of human hepatocellular liver carcinoma (Hep-G2) (IC_{50} = 1.9 µM), human skin melanoma (SK-Mel-28) (IC_{50} = 1.7 µM) and hepatoma (H-4-II) (IC_{50} = 2.7 µM). Therefore, it is possible to suggest potential antitumoral properties for triterpene 1.

The discovery and identification of new antitumor drugs with low side effects on immune system has become an essential goal in many studies of immunopharmacology (Zandi et al., 2010). Considering that constituent 1 is easily isolated from crude hexane/ethyl ether (1:1) extract from *M. gonoclada* root bark, in high yields, or from other species of the Celastraceae family, providing sufficient material to obtain new derivatives, it is feasible to get structural modifications at positions responsible for cytotoxicity, aiming a better activity and lower toxicity.

The present study signalizes the importance of semi-synthetic reactions to obtain derivatives of tingenone (compound 1) as promissory field, not only for the development of new drugs to treat amoebiasis and other diseases caused by protozoan, but also to obtain other alternative antitumoral drugs.

Conflict of Interest

The authors declare that no actual or potential conflict of interest exists in relation to this article.

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