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ORIGINAL ARTICLE

Antiprotozoal Constituents from *Annona cherimola* Miller, a Plant Used in Mexican Traditional Medicine for the Treatment of Diarrhea and Dysentery

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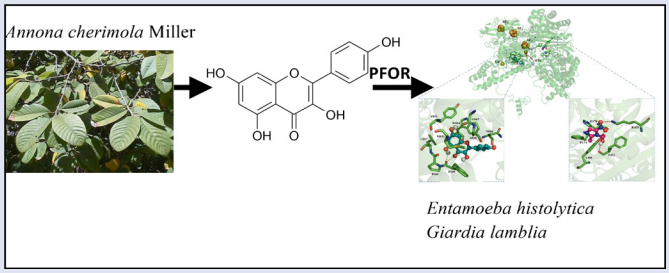
ABSTRACT

Background: *Annona cherimola* Miller (Annonaceae) is a medicinal plant frequently recommended in Mexican traditional medicine for the treatment of gastrointestinal disorders such as diarrhea and dysentery. **Objective:** This work was undertaken to obtain information that support the traditional use of *A. cherimola*, on pharmacological basis using *in vitro* and computational experiments. **Material and Methods:** Bioassay-guided fractionation of the ethanol extract of the leaves of *A. cherimola* afforded five phenolic compounds: caffeic acid, quercetin, kaempferol, nicotinflorin, and rutin. **Results:** The *in vitro* antiprotozoal assay showed that kaempferol was the most potent antiamoebic and antigiardial compound with IC_{50} values of 7.9 µg/mL for *Entamoeba histolytica* and 8.7 µg/mL for *Giardia lamblia*. Computational molecular docking study showed that kaempferol interacted in a region different than metronidazole in the enzyme pyruvate: ferredoxin oxidoreductase (PFOR). **Conclusion:** Considering that PFOR is a target of metronidazole; kaempferol may be a lead compound for the development of novel antiprotozoal agent. Also, these findings give support to the use of *A. cherimola* in the traditional medicine from México for the treatment of diarrhea and dysentery.

Key words: Annona cherimola, Annonaceae, Antiprotozoal activity, Piruvate: ferredoxin oxidoreductase, Giardia lamblia, kaempferol, Entamoeba histolytica

SUMMARY

Bioassay-guided fractionation of the ethanol extract of the leaves of *Annona cherimola* afforded five phenolic compounds: caffeic acid, quercetin, kaempferol, nicotinflorin and rutin. The *in vitro* antiprotozoal assay showed that kaempferol was the most potent antiamoebic and antigiardial compound with IC50 values of 7.9 µg/mL for *Entamoeba histolytica* and 8.7 µg/mL for *Giardia lamblia*. Computational molecular docking study showed that kaempferol interacted in a region different that metronidazole in the enzyme pyruvate: ferredoxin oxidoreductase.



Abbreviations used: PFOR: Pyruvate: ferredoxin oxidoreductase, *G: lamblia: Giardia lamblia, E: histolytica: Entamoeba histolytica*

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INTRODUCTION

Giardiasis and amoebiasis are enteric parasitic infections caused by anaerobic protozoa *Giardia lamblia* and *Entamoeba histolytica*, respectively. Symptomatic patients usually present diarrhea or dysentery together with abdominal symptoms such as stomach ache, abdominal cramps and bloating, both infections are a health problem in the world, particularly in the tropical and subtropical poor countries.[1-3] In the case of Mexico, amoebiasis is an endemic disease, with incidence rates that vary among the geographic regions of the country. In the last six years, it has been a serious health problem, and it was the 10th cause of morbidity among all age groups. In the case of giardiasis, it currently accounts for an estimated nine millions of sicks each year, and it is the leading cause of gastrointestinal parasitosis of medical importance in children.[4-7] There are numerous antiprotozoal drugs used in medical practice such as metronidazole, tinidazole, iodoquinol, diloxanidefuroate, and paromomycin. Among these metronidazol is the drug of choice currently used in Mexico for the treatment of infections caused by the flagellate *G. lamblia* and the amoeba *E. histolytica*. It is effective, however, is mutagenic in bacteria and carcinogenic in rodents. In addition, this drug has several other side effects including gastrointestinal disturbance, especially nausea and vomiting. Infrequent adverse effects include headache and stomatitis. Moreover, in long-term systemic treatment with metronidazole, patients experience dry mouth, metallic taste, headache, and vertigo. Also, it is associated with the development of leucopenia,

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neutropenia, and central nervous system toxicity. In addition, clinical and laboratory-generated resistant strains of both protozoa have been reported.[8-10] In an effort to improve the therapy for giardiasis and amoebic dysentery, new drugs that retain therapeutic efficacy and are devoid of adverse effects, should be developed. Medicinal plants used for the treatment of gastrointestinal diseases could be a source of such new drugs.

Annona cherimola Miller (Annonaceae) is an evergreen tree of 5 to 9 m of height with attractive leaf, it is ovate to elliptic, short blunt-pointed at the apex; dark green and slightly hairy on the upper surface. The plant is widely distributed in the subtropical and mild temperate zones of the world such as Peru, Ecuador, Bolivia, Chile, España, USA and México.[11] It is found in different parts of Mexico and ascribed local names include: "atís" (Michoacán), "tzontechkia" (Oaxaca), "lamatzapotl" (Puebla) and "yati" (Veracruz). The common names of this plant include "anono, chirimolla, chirimollo, palo de chirimoya and zapotecoron". In Mexican traditional medicine *A. cherimola* is used for the treatment of inflammation, fever, cough, cold, diabetes, and cancer. In particular, the leaves of the plant are used for the treatment of diseases of the gastrointestinal tract such as enteritis, vomiting, nausea, ulcers, indigestion, diarrhea and dysentery.[12,13] Previous chemical studies of *A. cherimola* led to isolation of alkaloids, flavonoids, sterols,[13,14] terpenoids,[15,16] cyclic peptides[17,18] and acetogenins.[19,20] Moreover, a survey of the literature on pharmacological investigations revealed that extracts of *A. cherimola* seeds have genotoxic, cytotoxic,[19,21] antiprotozoal,[22] antisecretory,[23] and antibacterial[13] properties. Also, it is a potent inhibitor of mitochondrial complex I.[20] The extracts of the leaves have anti hypercholesterolemic,[14] antidepressant, and anxiolytic activities.[15]

In view of these facts, this study was conducted to investigate the antiprotozoal activity of the ethanol extract, fractions and phenolic compounds obtained of the leaves of *A. cherimola* using *in vitro* and computational experiments.

MATERIAL AND METHODS

Plant material. The leaves from *A. cherimola* (Annonaceae) were collected by Dr. Fernando Calzada in February 2012 in San José, Tláhuac, D. F., México. The plant material was authenticated by MS Abigail Aguilar-Contreras of the Herbarium IMSSM of Instituto Mexicano del Seguro Social (IMSS) where the voucher specimenis conserved under reference number: 15795.

Extraction from A. cherimola. The air-dried and finely powdered leaves (2.0 kg) were extracted by maceration at room temperature with EtOH (2 times x20L). After filtration the extract were combined and evaporated *in vacuo*to yield 162.5 g of green residue.

Isolation and identification of phenolic compounds from the EtOH extract of A. cherimola. A portion of the ethanol extract (25.5 g) was suspended in ethanol-water (1:9; 60 mL) and successively partitioned with CH_2Cl_2 (DCM fraction,7.5 g, 100 mL x 2 times) followed by EtOAc (EtOAc fraction, 1.9 g, 100 mL x 2 times). The aqueous residual fraction (AR fraction, 15.7 g, 100 mL x 2 times) was lyophilized. DCM fraction (90 mg) was purified by preparative TLC (silica gel, EtOAc-EtOH, 90:10) to give caffeic acid (**1**, 27.0 mg). From the EtOAc fraction, rutin (530.0 mg) crystallized spontaneously, and then the residue from EtOAc fraction (90 mg) was resolved by preparative TLC (silica gel, EtOAc-EtOH-water, 90:16.5: 13.5) to give kaempferol (**3**, 9.0 mg), quercetin (**2**, 12.0 mg), nicotinflorin (**4**, 9.0 mg), and additional amounts of rutin (**5**, 15.0 mg).AR fraction (90 mg) was purified by preparative TLC (silica gel, EtOAc-EtOH, 90:10) to give additional amounts of rutin (36.0 mg) and nicotinflorin (18.0 mg). The structure of phenolic compounds **1**-**5** were ascertained by their physical and spectroscopic properties (MP, TLC, HPLC, and RMN) compared with those reported in the literature and with authentic samples (Sigma) available in our laboratory.

Antiprotozoal assays. *Entamoeba histolytica* strain HM1-IMSS used in all experiments was grown axenically at 37°C in TYI-S-33 medium supplemented with 10% heat inactivated bovine serum. In the case of *Giardia lamblia*, strain IMSS: 8909:1 was grown in TYI-S-33 modified medium supplemented with 10% calf serum and bovine bile. The trophozoites were axenically maintained and for assays were employed in the log phase of growth. *In vitro* susceptibility tests were performed using a subculture method previously described.[22] Briefly, *E. histolytica* (6×10^3) or *G. lamblia* (5×10^4) trophozoites were incubated for 48 h at 37°C in the presence of different concentrations (2.5-200 µg/mL) of the crude extract or pure compounds in dimethyl sulfoxide (DMSO). Each test included metronidazole (**6**, Sigma) a standard amoebicidal and giardicidal drug, a control (culture medium plus trophozoites and DMSO), and a blank (culture medium). After incubation, the trophozoites were detached by chilling and 50 µL samples of each tube were subcultured in fresh medium for another 48 h, without antiprotozoal samples. The final number of parasites was determined with a haemocytometer and the percentages of trophozoites growth inhibition were calculated by comparison with the control culture. The results were confirmed by a colorimetric method: the trophozoites, were washed and incubated for 45 min at 37°C in phosphate buffer saline with MTT (3-[4,5-dimethylhiazol-2-il]-2, 5-diphenyl tetrazolium bromide) and phenazinemethosulfate. The dye produced (formazan) was extracted and the absorbance was determined at 570 nm. The experiments were performed in duplicate for each protozoan and repeated at least three times. Data were analyzed using probit analysis.[24]

Statistical analysis. Data were analyzed using probit analysis. The percentage of trophozoites surviving was calculated by comparison with the growth in the control group. The plot of probit against log concentration was made; the best straight line was determined by regression analysis and the 50% inhibitory concentration (IC_{50}) values were calculated. The regression coefficient, its level of significance ($P<0.05$ indicates significant difference between group) and correlation coefficient were calculated and 95% CI values determined.[24,25]

Computational study. Considering that PFOR is a potential target for drug design against certain anaerobic pathogens[26,27] and that it is a target of metronidazole in anaerobic protozoa.[28,29] In order to investigate the location and the binding mode of kaempferol and to compare them with metronidazole, docking studies were carried out. To examine the interaction between kaempferol or metronidazole and PFOR,[26,27] docking simulations were done on the 3-D structure of enzyme. Molecular docking simulations were performed using version 4.2 of the program Auto Dock along with Auto Dock Tools using the hybrid Lamarckian Genetic Algorithm (LGA). This program was chosen because its algorithm allows full flexibility of small ligands as kaempferol and metronidazole, allowing it to find its optimal orientation/conformation in the PFOR. It has been shown that it successfully reproduces many crystal structure complexes and includes an empirical evaluation of the binding free energy. The preparation of PFOR and kaempferol input structure and the definition of the binding sites were carried out under a GRID-based procedure. First, a rectangular grid box was constructed overtoxin ($126 \times 126 \times 126$ Å^3). The set up of the grids were performed with 60 points in each dimension, with a spacing of 0.375 Å between the grid points. The X-ray coordinates used in the study were based on the structure of PFOR from *Desufovibrio africanus*. It was taken from the protein Data Bank (PDB ID: 1KEK; EC: 1.2.7.1).[27] All molecules of water were stripped prior to docking simulations and ions and thiamine di-phosphate were kept to conserve the catalytic activity of the enzyme. All docking simulations were carried out with LGA and were realized

Table 1: Antiprotozoal activity of the ethanol extract, fractions and pure phenolic compounds obtained from the leaves of *A. cherimola*[a]

	IC_{50} µg/mL (CI)[b]	
Compound	***E. histolytica***	***G. lamblia***
Ethanol extract	129.7(130.1-129.3)	137.3 (137.7-137.0)
DCM fraction	125.3(125.6-125.0)	131.7(132.1-131.5)
Caffeic acid **1**	199.1 (202.5-195.8)	28.8(28.9-28.6)
EtOAc fraction	27.2(127.4-126.9)	37.2(37.6-37.1)
Quercetin **2**	114.3(115.2-113.4)	26.5(28.3-22.1)
Kaempferol **3**	7.9 (8.0-7.8)	8.7(8.8-8.6)
AR fraction	124.4(124.7-124.0)	134.7(135.0-134.2)
Nicotinflorin **4**	30.9(31.0-30.8)	22.5(22.6-22.4)
Rutin **5**	119.7(121.1-118.5)	178.7(179.3-177.8)
Metronidazoled **6**	0.04 (0.10-0.03)	0.21 (0.27-0.14)

[a]Results are expressed as mean (n=6); [b]CI= 95% confidence intervals; [d]Positive control; *P < 0.05 compared to metronidazole; **Correlation coefficient >0.900

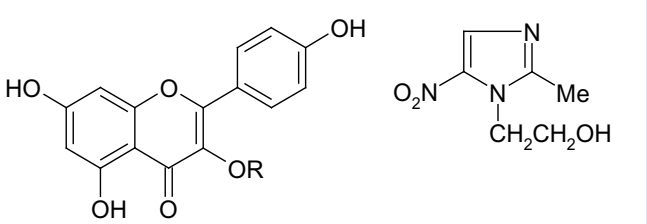

Figure 1: Chemical structures of kaempferol **3**, nicotinflorin **4** and metronidazole **6**. R=H= **3**; R=GlcORha= **4**

with an initial population of 100 randomly placed individuals and a maximum number of energy evaluations (1 x 10^7 cycles). Resulting docking orientations within 1.0 Å in the root-mean square deviation tolerance of each other were clustered together and represented by the result with the most favorable free energy of binding.

RESULTS AND DISCUSSION

Annona cherimola is a medicinal plant used in Mexico for the treatment of gastrointestinal diseases, including diarrhea and dysentery.[12,13] In the present study as part our research to obtain new antiprotozoal agents by medicinal plants and to rationalize their use in Mexican traditional medicine. Bioassay-guided fractionation of the active ethanol extract of the leaves of *Annona cherimola* on *E. histolytica* and *G. lambli*a was performed [Table 1]. The ethanol extract was fractionated into organic and aqueous soluble fractions by organic solvent extractions with DCM and EtOAc. All fractions (DCM, EtOAc and AR) were tested for antiprotozoal activity using *E. histolytica*and *G. lamblia* trophozoites. As result of this process, EtOAc-soluble fraction showed the best inhibitory activity on both protozoa, it was purified by preparative TLC to yield four flavonoids: kaempferol **3**, quercetin **2**, nicotinflorin **4** and rutin **5** [Figure 1]. In addition, the DCM and AR fractions were resolved by preparative TLC to yield a phenolic acid (caffeic acid, **1**) and additional amounts of the flavonoids, rutin and nicotinflorin.

The antiprotozoal activity of the flavonoids obtained in this work and caffeic acid were tested on *E. histolytica* and *G. lamblia* trophozoites [Table 1]. Of these phenolic compounds, kaempferol was the most potent compound on both protozoa with IC_{50} values of 7.9 µg/mL for

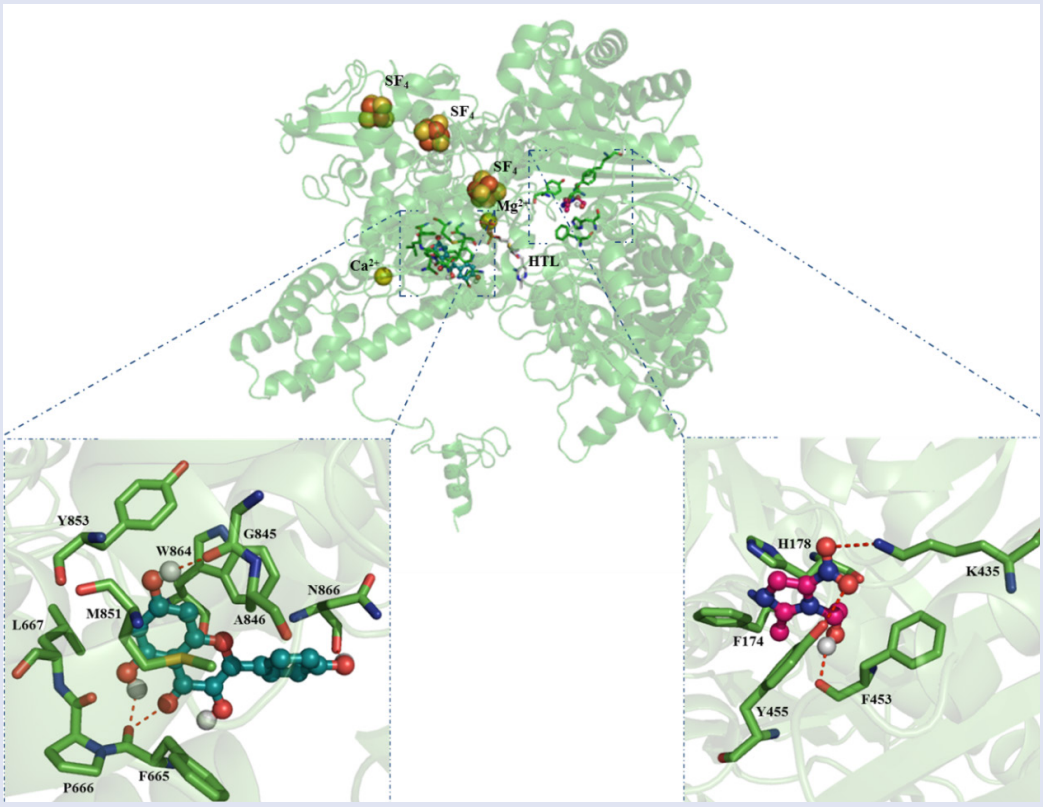

Figure 2: Are depicted the two binding sites onto PFOR of kaempferol (Lower left-panel) and metronidazole (Lower right-panel). H bonds are shown in red dashes. Y= Tyr; W=Trp, G=Gly, N=Asn; L=Leu, M=Met, A=Ala, P=Pro, F=Phe, H=His, K=Lys. SF_4= [4Fe-4S] cluster

E. histolytica and 8.7 μg/mL for *G. lamblia*. Nicotinflorin showed moderate activity on both organisms. The remaining phenolic compounds assayed showed weak activity on both protozoa. All compounds were less active than metronidazole, drug used as positive controls. To our knowledge, this is the first bioassay-guided work to obtain the antiprotozoal compounds of the leaves of *A. cherimola*.

Flavonoids are naturally occurring phenolic compounds that are found in medicinal plants and commonly consumed in the vegetables. Some flavonoids have been shown to display a number if interesting pharmacological activities, such as antioxidant,[30] antiprotozoal,[31] antiviral,[32] antipropulsive, and antisecretory activities.[33] In these sense, several flavonoids have been considered as the active principles of some antidiarrheic medicinal plants. It has been speculated that antidiarrheic properties are a consequence of their inhibitory actions on protozoa and intestinal peristalsis as demonstrated by *in vitro* and *in vivo* tests. Kaempferol is a flavonoid isolated of several medicinal plants with important *in vivo* and *in vitro* antigiardial and antiamoebic activity.[31,34,35] Also, nicotinflorin, which was obtained in EtOAc and AR fractions may be a prodrug of kaempferol considering that some flavonoids such as rutin are hydrolyzed by intestinal bacteria, cell-free extract from the small intestine, or gastrointestinal fluid.[36,39] Quercetin and quercetin glycosides isolated from *Psidium guajava* showed important spasmolytic effect on guinea-pig ileum.[36,40] In the case of kaempferol affects cytoskeleton in mammalian cell; in this context recently antiamoebic activity of this flavonoid was associated with deregulation of proteins related with cytoskeleton such as actin, myosin II heavy chain and cortexillin II. Also, in *E. histolytica*, it decreased adhesion, increased migration and phagocytic activity. Consequently, these data suggested that kaempferol could affect virulence properties of this human pathogen.[41]

The antiprotozoal activities of flavonoid glycoside, nicotinflorin and its aglycone kaempferol are in agreement with the results published by the author's group and confirm that kaempferol core may be a leading compound in the development of novel antiamoebic and antigiardial drugs.[31,34]

In relation with the molecular docking experiments is important to note that these showed as kaempferol interacted with nine amino acid residues, Phe 665 (at 3.74 Å), Pro 666 (at 4.00 Å), Leu 667 (at 3.65 Å), Gly 845 (at 3.39 Å), Ala 846 (at 4.38 Å), Met 851 (at 3.72 Å), Tyr 853 (at 3.32 Å), Trp 864 (at 4.54 Å) and Asn, 866 (at 3.98 Å) in a region different that metronidazole [Figure 2]. The binding energy of best scoring binding mode was calculated to be -9.60Kcal/mol, it was like to metronidazole. In the case of metronidazole have interactions with four amino acid residues Phe 174 (at 3.23 Å), His 178 (at 4.21 Å), Lys 435 (at 2.83 Å), Phe 453 (at 2.11 Å) and Tyr 455 (at 2.90 Å) with binding energy of -7.85 Kcal/mol. In agreement with docking analysis the antiprotozoal activity of kaempferol on anaerobic protozoa as *E. histolytica* and *G. lamblia* may be through of PFOR inhibition.

PFORs can be homo- or heterodimeric or heterotetrameric enzymes. They contain 1–3 iron-sulfur clusters and thiamine pyrophosphate as prosthetic groups. They are in anaerobic organisms (*E. histolytica* and *G. lamblia*) and catalyzed the oxidative decarboxylation of pyruvate to form acetyl-coenzyme A, with the transfer of a pair of electrons to ferredoxin; it process is a crucial step in many metabolic pathways as anaerobic glycolysis.[42] In presence of metronidazole the two electrons generated by the oxidative process are transferred to an iron-ferredoxin, which can then reduce metronidazole and activate it to toxic radical species.[29] Thus, PFOR is a potential target for drug design against certain anaerobic bacteria and protozoa such as *E. histolytica*, *G. lamblia* and *Trichomonasvaginalis*.[26,27] In these sense, since that kaempferol interacted in a region different that metronidazole in the enzyme PFOR, it should be considered as a leading compound in the developing of novel antiprotozoal agents in the control of diarrhea and dysentery caused by *G. lamlia*and *E. histolytica*.[28] In order to confirm the results obtained in computational experiments on the interaction between kaempferol and PFOR, proteomic approach based on two-dimensional gel electrophoresis and mass spectrometry (ESI-MS/MS) analysis, are in process.

Finally, results of the present work along with the properties previously describe from the extract and flavonoids obtained from *A. cherimola*, could suggest that the mechanism by which the plant inhibits the diarrhea and dysentery involves spasmolytic, antigiardial and antiamoebic effects. In this context, the antidiarrheic properties reputed for *A. cherimola* in Mexican traditional medicine may be due to presence of flavonoids: kaempferol, nicotinflorin, quercetin, and rutin.

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Nil

Conflicts of Interest

There are no conflicts of interest.

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