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ORIGINAL ARTICLE

Antiprotozoal, Antibacterial and Antidiarrheal Properties from the Flowers of *Chiranthodendron pentadactylon* and Isolated Flavonoids

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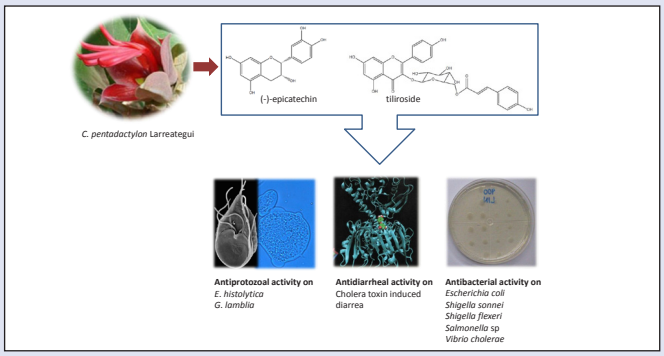
ABSTRACT

Background: *Chiranthodendron pentadactylon* Larreat. (Sterculiaceae) is a Mexican plant used in traditional medicine for the treatment of heart disease symptoms and infectious diarrhea. **Objective**: To evaluate *in vitro* antiprotozoal and antibacterial activities and *in vivo* antidiarrheal activity from the flowers of *C. pentadactylon* using the extract, fractions, and major isolated flavonoids. **Materials and methods**: Bioassay-guided fractionation of the methanol extract of *C. pentadactylon* (MECP) led to the isolation of five flavonoids, tiliroside, astragalin, isoquercitrin, (+)-catechin, and (-)-epicatechin. Antimicrobial activities were tested on two protozoa (*Entamoeba histolytica* and *Giardia lamblia*) and nine bacterial enteropathogens (two *Escherichia coli* strains, two *Shigella sonnei* strains, two *Shigella flexneri* strains, two *Salmonella* sp. strains, and *Vibrio cholerae*) isolated from feces of children with acute diarrhea or dysentery and resistant to chloramphenicol. Also, antidiarrheal activity was tested on cholera toxin-induced diarrhea in male Balb-c mice. **Results**: Epicatechin was the most potent antiamoebic and antigiardial compound with IC_{50} values of 1.9 µg/mL for *E. histolytica* and 1.6 µg/mL for *G. lamblia*; tiliroside showed moderate antiprotozoal activity against both protozoan. In contrast, in the antibacterial activity, tiliroside was the most potent compound on all microorganisms with minimum inhibitory concentration values less than 0.7 mg/mL. In the case of cholera toxin-induced diarrhea, epicatechin was the most potent flavonoid with IC_{50} of 14.7 mg/kg. **Conclusion**: Epicatechin and tiliroside were the flavonoids responsible for antimicrobial andantidiarrheal activities of *C. pentadactylon*. Its antiprotozoal, antibacterial, and antidiarrheal properties are in good agreement with the traditional medicinal use of *C. pentadactylon* for the treatment of infectious diarrhea.

Key words: *Chiranthodendron pentadactylon* Larreat, (-)-epicatechin, flavonoids, infectious diarrhea, Sterculiaceae, Tiliroside

SUMMARY

- Epicatechin was the most potent antiamoebic and antigiardial compound with IC_{50} values of 1.9 µg/mL for *E. histolytica* and 1.6 µg/mL for *G. lamblia*.
- Tiliroside showed antibacterial activity against all microorganisms tested with MIC values less than 0.7 mg/mL.
- Epicatechin was the most potent flavonoid on cholera toxin-induced diarrhea with IC_{50} of 14.7 mg/kg.



Abbreviations used: MECP: Methanol extract of *C. pentadactylon*

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INTRODUCTION

Worldwide gastrointestinal infections are the most common causes of diarrhea and are responsible for more deaths than gastrointestinal cancers, peptic ulcer, or inflammatory bowel disease. Diarrheal diseases are responsible for 1.5 million deaths every year in low- and middle-income countries, affecting principally children younger than 5 years.[1-3] There are an estimated 2.5 billion episodes of childhood diarrhea each year.[1,2] In México, during the past 10 years gastrointestinal infections have been a serious health problem and were the second common cause of morbidity among all age groups. There are vast numbers of bacteria, viruses, and parasites that can cause diarrheal disease, including

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Escherichia coli, *Salmonella* spp., *Shigella* spp., *Clostridium difficile*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Vibrio cholerae*, rotavirus, calicivirus, astrovirus, *Giardia lamblia*, and *Entamoeba histolytica*.[4,5]

Chiranthodendron pentadactylon is a medicinal plant that grows in Mexico. It is known with the name "flor de manita" in the States of Guerrero, Morelos, Puebla, and Veracruz; its flowers are used to treat mainly heart illness, epilepsy, and as anecdotic remedy for infectious diarrhea.[6-8] Because of its popularity, pharmacologic and phytochemical studies have been conducted by Calzada and collaborators since 2005 on *C. pentadactylon*. Briefly, MeOH extracts from the flowers of "flor de manita" had antibacterial, antiprotozoal, vasorelaxant, antisecretory, and antipropulsive properties.[9-13] Also, MeOH extract does not cause toxicity signs and death a dose of 4.8 g/kg p.o.[14] Phytochemical studies on *C. pentadactylon* have revealed the presence of hydrocarbons, steroids, sugars, and flavonoids.[6,14,15] Previous studies on antisecretory activity revealed that (-)-epicatechin exerted antisecretory activity through *Vibrio cholera* toxin inhibition.[14,16] Although antimicrobial and antipropulsive properties of MeOH extract of *C. pentadactylon* have been described, bioassay-guided studies to isolate its antiprotozoal, antibacterial, and antidiarrheal constituents have not been experimentally explored. The aim of this work was to evaluate antimicrobial and antidiarrheal activities from the flowers of *C. pentadactylon* using the major isolated flavonoids to obtain additional information that support the anecdotic use of "flor de manita" as an agent for the treatment of infectious diarrhea in Mexican traditional medicine.

MATERIALS AND METHODS

General experimental procedures

IR spectra were recorded as KBr pellets on a Perkin-Elmer 599 B spectrophotometer. 1H-NMR (300 MHz) and 13C-NMR (75 MHz) spectra were registered on a Varian VXR-300S spectrometer in $CDCl_3$ with TMS as internal standard. The chemical shifts are reported in δ units (ppm). EI-MS were taken on a Hewlett-Packard 5985 apparatus, at ionization energy of 70 eV. Melting points were determined in a Fischer Johns apparatus and are uncorrected. Optical rotations were taken on a digital polarimeter JASCO Dip 360. CC: silica gel 60 (Merck, 30-70 mesh). TLC: 0.25 mm or 1 mm precoated silica gel60 F_{254} (Merck).

Plant material

The flowers from *C. pentadactylon* (Sterculiaceae) were collected by Dr. Fernando Calzada in October 2014 in Ozumba, State of Mexico, Mexico. The plant material was authenticated by MS Abigail Aguilar-Contreras, of the Herbarium IMSSM of Instituto Mexicano del Seguro Social (IMSS) where the voucher specimen (No. 14404) was deposited.

Extraction from *C. pentadactylon*

The air-dried flowers (1.0 kg) were ground and extracted by maceration at room temperature with MeOH (2 L × two times). After filtration, the solvent was evaporated in vacuo to yield 142 g of red extract (13.8%).

Isolation of flavonoids from the flowers of *C. pentadactylon*

Flavonoids were isolated from the MeOH extract from the flowers of *C. pentadactylon* according to the method of Velázquez.[14] Briefly, the MeOH active extract (40 g) was suspended in 10% MeOH–water (70 mL) and successively partitioned with CH_2Cl_2 (70 mL × three times, 3.0 g) and EtOAc (70 mL × three times, 1.5 g). The aqueous residual layer (ARL, 1.9 g) was lyophilized. A portion of the EtOAc fraction (500 mg) was purified by preparative TLC (Merck, TLC silica gel 60 F_{254}, EtOAc-MeOH–H_2O, 100:16.5:13.5) to give astragalin (yellow powder, MP 204-205°C, 20.0 mg), tiliroside (yellow powder, MP 207-208°C, 25.0 mg), (+)-catechin (brown light powder, MP 182-185°C, 18.3 mg), (-)-catechin (brown light powder, MP 242-244°C, 21.7 mg) and isoquercitrin (yellow powder, MP 187–188°C, 23.3 mg). The compounds were characterized by the reported ^{13}C and ^{1}H NMR data, as well as by direct comparison of TLC and MP with authentic samples available in our laboratory.[14]

Antiprotozoal assays

E. 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 *G. 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 assays were employed in the log phase of growth. *In vitro* susceptibility tests were performed using a subculture method previously described.[17] 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 a control (culture medium plus trophozoites and DMSO), a blank (culture medium), metronidazole (Sigma) and emetine (Sigma) as amoebicidal and giardicidal drugs. 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 hemocytometer 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 phenazine methosulfate. 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.[18] 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), and correlation coefficient were calculated and 95% CI values determined.

Antibacterial testing

The samples were tested against nine microorganisms, two *Escherichia coli* species, two *Shigella sonnei* species, and two *Shigella flexneri* species, two *Salmonella* sp. species, and *Vibrio cholerae*. The bacterial inoculum of each strain was obtained from fresh colonies grown on Müller-Hinton agar plates (MHA, Sigma). All bacterial strains used in this study were isolated from the feces of children with acute diarrhea or bloody diarrhea.[19] Also, all strains tested are resistant to chloramphenicol. The minimum inhibitory concentration (MIC) of samples was accurately determined by agar dilution technique.[10] Briefly, the extract, fractions, and pure compounds for testing were dissolved in DMSO and serially diluted in melted MHA plates (100 mm × 15 mm) to obtain the final concentrations: 0.5, 1, 2, 4, and 8 mg/mL (extract and fractions) or 100-2000 μg/mL (pure compounds). The solvent did not exceed 1% concentration and did not affect the growth of any of the microorganisms. The cultures were diluted in Müller–Hinton broth (MHB, sigma) at a density adjusted to a 0.5 McFarland turbity standard (1.5×10^8 colony-forming units [CFU]/mL). The inoculum was added to a Steer's replicator calibrated to deliver 10^4 CFU. Then Petri dishes were inoculated and incubated at 37°C, examined after 24 h, and further incubated for 72 h. Chloramphenicol

Table 1: Antiprotozoal, antibacterial, and antidiarrheal activities of the MeOH extract, active fraction, and flavonoids from *C. pentadactylon*

	IC_{50} (μg/mL)[a]		MIC (mg/mL)[b]									IC_{50} (mg/mL)[c]
Compounds	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
MeOH extract	106.7 (107.0-106.3)	120.9(121.0-120.8)	2	2	2	2	2	2	2	2	2	351 ± 1.2
EtOAc fraction	20.3 (20.5-20.0)	34.3 (34.6-34.1)	1	0.5	1	1	0.5	0.5	1	1	1	217 ± 1.1
(-)- Epicatechin	1.9 (2.0-1.3)	1.6 (1.7-1.4)	>2	>2	>2	>2	>2	>2	>2	>2	>2	14.7 ± 1.01
Tiliroside	17.5 (22.1-116.5)	17.4 (22.3-13.5)	0.7	0.5	0.6	0.6	0.5	0.6	0.6	0.6	0.6	>120
Isoquercitrin	14.7 (14.9-14.5)	87.3 (87.4-87.2)	>2	>2	>2	>2	>2	>2	>2	>2	>2	>120
Astragalin	61.2 (61.5-61.0)	47.5 (47.6-47.4)	>2	>2	>2	>2	>2	>2	>2	>2	>2	>120
(+)-Catechin	65.6 (67.3-62.2)	34.0 (35.1-31.5)	>2	>2	>2	>2	>2	>2	>2	>2	>2	>120
Metronidazole[d]	0.04 (0.10-0.03)	0.21 (0.27-0.14)	-	-	-	-	-	-	-	-	-	-
Emetine[d]	1.05 (1.06-1.03)	0.41 (0.42-0.40)	-	-	-	-	-	-	-	-	-	-
Chloramphenicol[d]	-	-	>2	>2	>2	>2	>2	>2	>2	>2	>2	-
Rececadotril[d]	-	-	-	-	-	-	-	-	-	-	-	71.8 ± 2.7

A: *Entamoeba histolytica*, B: *Giardia lamblia*, C: *Escherichia coli*-1, D: *E. coli*-2, E: *Shigella sonnei*-1, F: *S. sonnei*-2, G: *S. flexneri*-1, H: *S. flexneri*-2, I: *Salmonella* sp-1, J: *Salmonella* sp-2, K: *Vibrio cholerae*-1, and L: cholera toxin induced-diarrhea. [a]Results are expressed as mean ($n = 6$), CI = 95 % confidence intervals, $P < 0.05$, Correlation coefficient > 0.9500. [b]Results are expressed as mean ($n = 4$). [c]Results are expressed as mean ($n = 6$) ± S.E.M., $P < 0.05$, Correlation coefficient > 0.9700. [d]Drug control

(Sigma) was used as reference standard and for comparative purpose. The lowest concentration of the sample in a plate that failed to show any visible macroscopic bacterial growth was considered as the MIC. The MIC determination was performed in duplicate for each organism, and the experiment was repeated two times.

Animals

Male Balb-c mice were obtained from animal house of the National Medical Center "Siglo XXI" from Mexican Institute of Social Security (IMSS). Investigations using experimental animals were conducted in accordance by the Official Mexican Rule.[20] They were maintained in a temperature room (22 ± 2°C) on a 12-h light–dark natural cycle. Mice were fed with standard diet and water ad libitum. These studies were conducted with the approval of the Speciality Hospital Bio-Ethical Committee of the National Medical Center "Siglo XXI" from IMSS (Approval No.: R-2012-3601-182).

Cholera toxin

Lyophilized powder (1 mg) of cholera toxin (Sigma) containing approximately 220,000 units/mg of protein was suspended in 5 mL of sterile water. For the study, aliquots of the toxin solutions were dissolved in sterile water (0.5 mL) to obtain a concentration of 10 μg/mL.

Cholera toxin-induced diarrhea

Male Balb-c mice (20-22 g, 8 weeks old) were randomly allocated in six groups ($n = 6$), with free access to water. Diarrhea was induced in the experimental groups by cholera toxin (0.5 mL of cholera toxin a concentration of 10 μg/mL × mouse) except of blank. After 30 min, materials were administrated as follows: blank (0.5 mL of 2% DMSO solution in water), positive control (0.5 mL of 2% DMSO solution in water), MECP (200, 300, 350, and 700 mg/kg in 0.5 mL of a 2% DMSO in water), fractions (200, 300, 350, and 700 mg/kg in 0.5 mL of a 2% DMSO in water), racecadotril (Ferrer Internacional, Hidrasec, 100 mg) was used as antidiarrheal agent (10, 20, 40, 80, and 120 mg/kg in 0.5 mL of a 2% DMSO in water), and flavonoids (10, 20, 40, 80, and 120 mg/kg in 0.5 mL of a 2% DMSO in water). All test materials and cholera toxin were administered intragastrically. Immediately after administration, the animals were placed in cages lined with adsorbent paper and were observed for 4 h then the total mass of fecal output (mg) was measured and expressed in percentage of inhibition.

Statistical analysis

After the plot of percentage of inhibition against 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), and correlation coefficient were calculated. The experiments were performed two times (six animals each times) for each concentration. IC_{50} values are mean ± S.E.M. P was less than 0.05 (differences between groups were analyzed by one-way ANOVA followed by Dunnett and Bonferroni posthoc test). GraphPad Prism Version 5.03 was used.

RESULTS

Active compounds of *C. penthadatylon*

In the present work, the flowers *C. pentadactylon* were extracted with MeOH. The MeOH extract was fractionated into fractions of different polarity by organic solvent extractions with CH_2Cl_2 and EtOAc. All fractions (CH_2Cl_2, EtOAc, and ARL) were tested for *in vitro* antiprotozoal and antibacterial activities and the *in vivo* antidiarrheal activity in mice [Table 1]. As the EtOAc-soluble fraction showed the best antimicrobial and antidiarrheal inhibitory activities, it was purified by preparative TLC to yield three flavonol glycosides and two flavan-3-ols. These compounds were identified as astragalin, tiliroside, isoquercitrin, (–)-epicatechin, and (+)-catechin [Figure 1] The CH_2Cl_2 and ARL fractions were discarded because none of them showed activity in all the tests.

Antiprotozoal, antibacterial and antidiarrheal activities

The results obtained confirm that the methanol extract is effective on all enteropathogens tested and cholera toxin-induced diarrhea [Table 1]. The MeOH extract exhibited antiprotozoal activity against both protozoa with IC_{50} values of 106.7 μg/mL for *E. histolytica* and 120.9 μg/mL for *G. lamblia*. In the case of antibacterial activity, it showed effect on all bacterial strains with MIC values 2 mg/mL. Thus, the activity of the Me OH extract from *C. pentadactylon* was superior to that chloramphenicol (MIC values > 2 mg/mL). In addition, antidiarrheal activity of MeOH extract was tested on cholera toxin-induced diarrhea in mice model and showed inhibitory effect with an IC_{50} of 351 mg/kg. Its antidiarrheal property is in agreement with the antisecretory activity previously reported to the MeOH extract of *C. pentadactylon*.[14] Further chemical fractionation indicated that antimicrobial and antidiarrheal properties

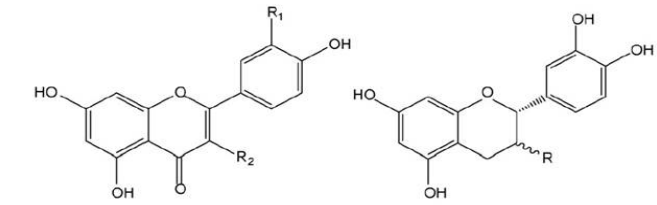

Figure 1. Isolated flavonoids from the flowers of *Chiranthodendron pentadactylon*

Compound	R_1	R_2
Tiliroside	H	*O*-(6′′-*O*-*E*-*p*-coumaroyl)-*B*-*D*-glucopyranoside
Astragalin	H	*O*-*B*-*D*-glucopyranoside
Isoquercitrin	OH	*O*-*B*-*D*-glucopyranoside
(+)-catechin	R= ◀ OH	
(-)-epicatechin	R= ····· OH	

Figure 1: Isolated flavonoids from the flowers of *C. pentadactylon*

were associated with the EtOAc-soluble fractions. Then (-)-epicatechin was isolated as the most potent antiamoebic and antigiardial compound with IC_{50} values of 1.9 μg/mL for *E. histolytica* and 1.6 μg/mL for *G. lamblia*; tiliroside showed moderate antiprotozoal activity against both protozoan. The antiprotozoal activities of epicatechin and tiliroside are in agreement with the results published the author's group and confirm that both flavonoids may be a leading compounds in the development of antiamoebic and antigiardial drugs.[21,22] (In contrast, the antibacterial activity, tiliroside was the most potent compound on all microorganisms with MIC values < 0.7 mg/mL.) The antibacterial activity of tiliroside was superior to that of chloramphenicol. In the case of cholera toxin-induced diarrhea, (-)-epicatechin was the most potent flavonoid with IC_{50} of 14.7 mg/kg. Its effect was superior to that of racecadotril (IC_{50} of 71.8 mg/kg) used as antidiarrheal agent. Of the remaining flavonoids, none of them exhibited antidiarrheal activity in the model used at concentrations lower than 120 mg/kg. (-)-Epicatechin and tiliroside were the flavonoids responsible for *in vitro* and *in vivo* activities of *C. pentadactylon*. Its antidiarrheal properties are in good agreement with the traditional medicinal use of *C. pentadactylon* for the treatment of infectious diarrhea.

DISCUSSION

In developing countries, infectious diarrhea is a major cause of mortality and morbidity. In this sense, medicinal plants with traditional use have been an important source of new biologically active molecules. In spite of the use of *C. pentadactylon* in Mexican traditional medicine for the treatment of infectious diarrhea and that antimicrobial and antipropulsive properties of MeOH extract of *C. pentadactylon* have been described, bioassay-guided studies to isolate its antiprotozoal, antibacterial, and antidiarrheal constituents have not been experimentally explored. In the present study, we demonstrate that MeOH extract and its flavonoids have antimicrobial activity and that this activity is important on enteropathogens associated with severe bloody diarrhea (*Shigella* spp.) and with severe watery diarrhea (*Vibrio cholerae*). The spectrum of antimicrobial activity of this plant is very important considering the episodes associated with *Shigella*, *V. cholera*, and *E. histolytica* in Mexico.[5] In Mexico, people use one or two handfuls of flowers and drink a cup of tea for 4 days without side effects, which is in agreement with the results reported by Velázquez *et al.*[14] Extract, EtOAc fraction, and tiliroside were effective on bacterial isolates with low sensitivity to chloramphenicol. The use of chloramphenicol is no longer recommended because of the widespread resistance to it. It is important to note that the effect of tiliroside was superior to chloramphenicol. In summary, the results of the present study along with the properties previously described from the extract and flavonoids obtained from *C. pentadactylon* could suggest that the mechanism by which the flowers inhibit the infectious diarrhea such as secretory diarrhea and dysentery involves antiamoebic, antigiardial, antibacterial, antisecretory, spasmolytic, antipropulsive, and anti-cholera toxin-induced diarrhea. Finally, the results open the possibility of investigating the use of the flowers from *C. pentadactylon* as a therapeutic source for the treatment of infectious diarrheal diseases.

CONCLUSION

(-)-Epicatechin and tiliroside were the flavonoids responsible for antimicrobial and antidiarrheal activities of *C. pentadactylon*. Its antiprotozoal, antibacterial, and antidiarrheal properties are in good agreement with the traditional medicinal use of *C. pentadactylon* for the treatment of infectious diarrhea.

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Conflicts of interest

There are no conflicts of interest

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