Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Phytochemical Analysis, Antipropulsive and Antilymphoma Activities of Leaves Extract of *Annona cherimola* Miller

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Submitted: 18-Oct-2019 Revised: 06-Dec-2019 Accepted: 16-Dec-2019 Published: 15-Jun-2020

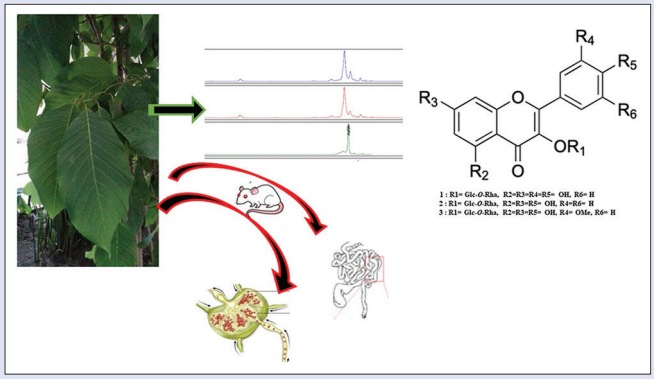
ABSTRACT

Background: Diarrhea and cancer are significant health problems worldwide. *Annona cherimola* Mill. (Annonaceae) is a fruit tree extensively used in Mexican traditional medicine for the treatment of diarrhea and cancer. **Objectives:** The present work reports the identification of flavonol glycosides present in the ethanol crude extract using high-performance liquid chromatography with diode array detection (HPLC-DAD). **Materials and Methods:** The identification of flavonoids was made by comparing their retention times and ultraviolet spectra with those standards. The crude extract, dichloromethane, and aqueous residual (AR) fractions were tested for their antipropulsive and antilymphoma activities. **Results:** The results revealed high levels of three flavonoid glycosides (rutin, nicotiflorin, and narcissin), especially rutin (24.5 mg/g of extract). The antipropulsive and antilymphoma activities of the leaf extract and subsequent fractions showed that the ethanol extract of the leaves of *A. cherimola* is cursive and AR fraction exhibited the best biological effects in both assays. **Conclusion:** The HPLC-DAD method used in this work enables the determination of rutin as a major flavonol glycoside of the leaves of *A. cherimola*. In addition, it can be suggested that rutin may play an important role in the biological properties of *A. cherimola.*

Key words: *Annona cherimola*, cancer, diarrhea, flavonol glycosides, high-performance liquid chromatography with diode array detection

SUMMARY

- The present work reports the identification of flavonol glycosides present in the ethanol crude extract from *Annona cherimola* using high-performance liquid chromatography with diode array detection (HPLC-DAD). In addition, the ethanol extract of the leaves of *A. cherimola* and subsequent fractions were evaluated for their antipropulsive and antilymphoma activities. The results of HPLC-DAD revealed high levels of rutin, nicotiflorin, and narcissin. In addition, it can be suggested that rutin may play an important role in the antipopulsive and antilymphoma properties of *A. cherimola.*



Abbreviations used: DAD: Diode array detection; HPLC: High-performance liquid chromatography; NHL: Non-Hodgkin lymphomas; IMSS: Instituto Mexicano del Seguro Social; EEL*Ac*: Ethanol extract of the leaves of *Annona cherimola*; DCM: Dichloromethane; AR: Aqueous residual.

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E-mail: fercalber10@gmail.com
DOI: 10.4103/pm.pm_462_19

Access this article online
Website: www.phcog.com
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INTRODUCTION

Diarrhea and cancer are significant health problems worldwide including Mexico. Diarrhea is defined as the passage of three or more loose or liquid stools per day, which mostly results from contaminated food and water sources. According to the World Health Organization, diarrheal diseases are the second leading cause of death in children under 5 years old around the world, and is responsible for around 525,000 deaths of children every year.[1] In Mexico, 3.4/100,000 people died due to diarrhea in 2016.[2] Non-Hodgkin lymphomas (NHL) are a heterogeneous group of malignancies of the lymphoid system. It is a cancer that affects the lymph nodes or other lymph tissue, which originates from the malignant transformation of B and T lymphocytes. NHL affects adults and children. In 2015, over 4.3 million people had NHL, with 231,400 deaths around the world.[3] In Mexico, from 2011 to 2016, NHL was the third most common cancer in males and the sixth among females.[4]

Annona cherimola Mill. (Annonaceae) is a medicinal plant known in Mexico as chirimoya. Its fruit is used as food and its leaves have proven

Cite this article as: Calzada F, Valdés M, Merlin VI, Velázquez C, Barbosa E, Chávez-Soto M, *et al*. Phytochemical analysis, antipropulsive and antilymphoma activities of leaves extract of *Annona cherimola* Miller. Phcog Mag 2020;16:317-20.

to be a remedy for the treatment of diarrhea, dysentery, abdominal pain, diabetes, and cancer.[5,6] The phytochemical investigations of *A. cherimola* led to the isolation of flavonoids, alkaloids, terpenoids, cyclic peptides, acetogenins, amino acids, organic acids, carbohydrates, phenolic acid derivatives, cholines, and phenylpropanoids. Biological studies have shown that extracts of *A. cherimola* seeds have genotoxic, cytotoxic, antiprotozoal, inhibitor of mitochondrial complex I, antibacterial, apoptotic, and antisecretory properties. Moreover, the extracts of the leaves have antihypercholesterolemic, anxiolytic, antidepressant, antihyperglycemic, and antiprotozoal activities. Recently, two bioassay-guided studies have been conducted on *A. cherimola* collected in the Cd Mexico. From the ethanol extracts of the leaves of *A. cherimola,* five phenolic compounds (quercetin, kaempferol, nicotiflorin, rutin, and caffeic acid) were isolated, among which kaempferol was the main antiprotozoal compound. Another study led to the identification of rutin as the major antihyperglycemic compound.[7-11]

MATERIALS AND METHODS

Chemicals

The standards rutin 1, nicotiflorin 2, and narcissin 3 as well as acetonitrile, ethanol, and acetic acid of high-performance liquid chromatography (HPLC) grade were acquired from Sigma (St Louis, MO, USA). In all cases, the water used was of HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA).

Plant material

A. cherimola Mill (Annonaceae) was collected by Dr. Edwin Cruz in July 2018 in Tultitlán (19°38'06.3"S 57°05'50.5"W) State of Mexico, Mexico. The leaves were authenticated by MS Santiago Xolalpa, taxonomist of the Instituto Mexicano del Seguro Social (IMSS). A voucher specimen has been preserved with an accession number 16098 in the institutional herbarium.

Extraction and fractionation from *Annona cherimola*

In order to obtain an ethanol crude extract, the air-dried and finely powdered leaves (3 kg) were extracted by maceration at room temperature (22°C ± 2°C) in absolute ethanol (10 L) twice for 1 week, yielding 135 g (4.5%) of the ethanol extract of the leaves of *A. cherimola* (EEL*Ac*) as green residue. The EEL*Ac* (50 g) was suspended in ethanol–water solution (1:9; 100 mL) and partitioned twice with dichloromethane (DCM) (100 mL) to yield DCM fraction (43.8 g). The aqueous residual fraction (AR fraction) was concentrated under reduced pressure to dryness to yield 18.9 g.

Determination of flavonol glycosides by high-performance liquid chromatography

The EEL*Ac* and the AR fraction were analyzed by HPLC-diode array detection (DAD). Analyses were conducted in a Waters 2795 liquid chromatograph system coupled with a Waters 996 photodiode array detector and an analytical Millennium 3.1 workstation as well equipped with a C_{18} column (200 mm × 15 mm ID, particle size of 5 µm (Spherisorb S5ODS2, Waters Corporation, Milford, MA, USA). For elution, a binary mobile phase consisting of (A) water containing acetic acid (2%) and (B) acetonitrile was used as follows: 1st stage – linear gradient of solvents A and B (from 96% to 88% of A) for 20 min; 2nd stage – linear gradient of solvents A and B (from 88% to 80% of A) 10 min; 3rd stage – linear gradient of solvents A and B (from 80% to 50% of A) 15 min; 4th stage – linear gradient of solvents (from 50% to 96% of A) for 15 min with a flow rate of 1 mL/min of mobile phase. In all the analyses, the injected sample volumes, prepared in ethanol, were 20 µL wavelength of 254 nm, and the data collected were plotted. The standards rutin 1, nicotiflorin 2 and narcissin 3 as well as acetonitrile, ethanol and acetic acid of HPLC grade were acquired from Sigma (St Louis, MO, USA). In all cases, the water used was of HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). Extract, AR fraction, and flavonoids were filtered through 0.45 µm.

Animals

Sprague-Dawley rats (3 months old; 250–300 g), Balb-c male mice (25 ± 4 g), and CD-1 male mice (30 ± 5 g) were obtained from the Animal House of the National Medicinal Center "Siglo XXI" from IMSS. Investigations were conducted in accordance with the Official Mexican Rule (NOM-062-ZOO, 1999), guaranteeing the welfare of experimental animals and with the approval of the Speciality Hospital Ethical Committee from IMSS (Register: R-2017-3601-217, R-2019-3601-004, R-2019-3601-024, and R-2019-3601-227). The rodents were maintained at room temperature (22°C ± 2°C) on a 12h light-dark natural cycle.[12]

Determination of the effect of ethanol extract of the leaves of *Annona cherimola* and subsequent fractions on charcoal-gum acacia-induced hyperperistalsis in rats

The effects of EEL*Ac* and its fractions on hyperperistalsis in male rats were tested using previously described procedures. The rats were divided into groups (n = 6, per group). The rats were treated orally with each sample (0.1 to 100 mg/kg in 1 mL), vehicle (1 mL of a 2% DMSO solution in water), or loperamide hydrochloride (Sigma), as positive control. After 20 min, each of the animals was given 1 mL of charcoal meal (10% charcoal suspension in 5% aqueous Arabic gum) by oral route. All animals were sacrificed after 30 min, the distance moved by the charcoal meal from the pylorus was measured, and the 50% inhibitory concentration (IC_{50}) values were calculated.[13]

Determination of the effect of ethanol extract of the leaves of *Annona cherimola* and subsequent fractions on induced lymphoma mouse model

The human lymphoma U-937 cell line (American Type Culture Collection: CRL 1593.2) was used in *in vivo* tests. The cells (2.0×10^6/mL) were seeded in (Roswell Park Memorial Institute) RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum and incubated at 37°C with 5% CO_2. The antilymphoma activity was evaluated using previously described procedures. The CD-1 male mice were divided into groups (n = 6, per group). For comparison, a group of healthy mice was included. NHL was induced by one intraperitoneal injection with 1×10^6 U-937 cells/mouse. After 24 h, the animals were treated for 9 days with the extract (50–150 mg/kg) or fractions (50–150 mg/kg) or methotrexate (0.5–1.5 mg/kg) orally. The animals were maintained under observation for 30 days, and the number of surviving animals was recorded daily. After the completion of the experiment, all animals were sacrificed in a CO_2 chamber, and the axillary and inguinal lymph nodes were removed and weighed. The 50% effective inhibitory concentration (EC_{50}) values were calculated.[14]

Statistical analysis

The plot of percentage of inhibition against concentration was made; the best straight line was determined by regression analysis, and the EC_{50} or IC_{50} was calculated. All data were expressed as mean ± standard deviation of six measurements. Statistical analysis of data was performed using one-way analysis of variance. $P < 0.05$ was considered

statistically significant. Differences between groups were analyzed by Dunnett *post hoc* test. Analyses were performed using GraphPad Prism Version 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

Figures 1 and 2 show the chromatograms of the EEL*Ac*, AR fraction, and flavonoid glycosides (1–3). Table 1 lists the retention times of each flavonoid glycoside (1–3), as well as ultraviolet (UV) absorption maxima of each peak obtained by DAD. The gradient elution method used allowed a good separation of the major flavonoid glycosides present. Peaks with retention times up to 32.645, 33.497, and 35.052 min exhibited UV characteristics identical with those of flavonol glycosides, and these were identified in the leaves of *A. cherimola* as rutin 1 (24.5 mg/g of the ethanol extract), nicotiflorin 2 (4.98 mg/g of the ethanol extract), and narcissin 3 (1.036 mg/g of the ethanol extract). The HPLC-DAD method used in this work enabled the quantitative determination of the flavonol glycosides present in the leaves of *A. cherimola.* These results agree with the other results reported and confirm that rutin is a major flavonol glycoside in the extract and most polar fractions of *Annona* species.[7,8,11,15] It is important to point that a recent work was reported in the leaves of *A. cherimola* collected in Spain a concentration of rutin of 12.31 mg/g of EEL*Ac* using HPLC-ESI-TOF-MS and NMR method. Comparing the results achieved in this work, the leaves of *A. cherimola* demonstrated to have the most highest rutin content (24.5 mg of 1/g of ethanol extract).[11] The presence of flavonol glycosides as rutin 1, in several plants used traditionally for the treatment of diarrhea, dysentery, diabetes, and cancer including *Annona* species, could account for the effectiveness of such plants in traditional medicine as therapeutic agents. The EEL*Ac* and AR fraction exhibited the best antipropulsive and antilymphoma effects [Table 2]. In this sense, a large amount of rutin 3 was detectable in EEL*Ac* and AcR fractions; it may contribute in the antipropulsive and antitumor properties of the leaves of *A. cherimola* considering that flavonol glycoside has been reported to have potent antipropulsive and anticancer effects associated with several mechanisms of action.[7-10,14] In this sense, the results of the present work along with the antiprotozoal properties previously described from the EEL*Ac* could suggest that mechanism by which the plant inhibits diarrhea involves antiprotozoal and spasmolytic effects.[7] In this context, the antidiarrheic

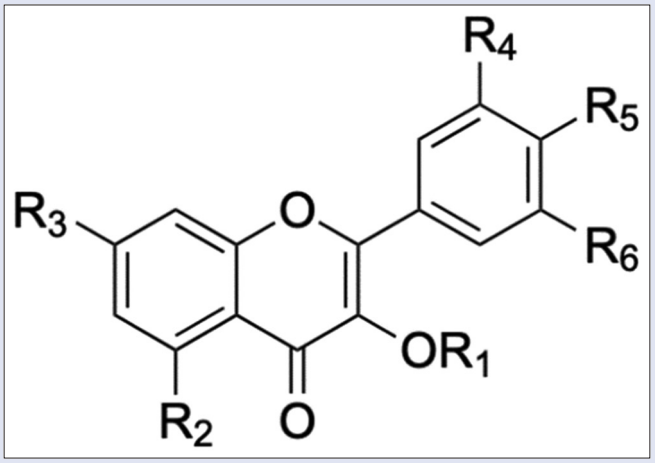

Figure 1: Chemical structure of rutin 1, nicotiflorin 2, and narcissin 3 identified in *Annona cherimola* leaf extract. (1) R1 = Glc-*O*-Rha, R2 = R3 = R4 = R5 = OH, R6 = H, (2) R1 = Glc-*O*-Rha, R2 = R3 = R5 = OH, R4 = R6 = H, (3) R1 = Glc-*O*-Rha, R2 = R3 = R5 = OH, R4 = OMe, R6 = H

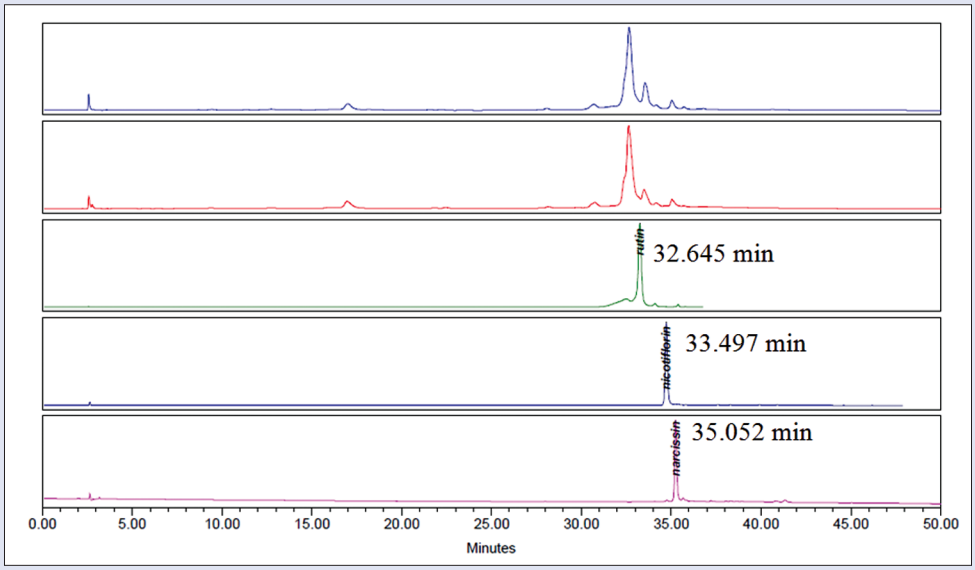

Figure 2: High-performance liquid chromatography with diode array detection analysis at 254 nm of the ethanol extract of the leaves from *Annona cherimola* (blue), Aqueous residual fraction (red), rutin (green), nicotiflorin (black), and narcissin (pink)

Table 1: Retention times and ultraviolet absorbance maxima of the major flavonol glycosides from *Annona cherimola*

Peak number	Analyte	λ_{max} (nm)	t_R (min)	mg of compound/g of ethanol extract	mg of compound/g of AcR fraction
1	Rutin	225.8, 254.4, 353.4	32.645	24.5	43.18
2	Nicotiflorin	228.6, 255.4, 353.6	33.497	4.98	5.99
3	Narcissin	229.8, 265.0, 346.8	35.052	1.036	1.88

AcR: Aqueous residual fraction

Table 2: Antipropulsive and antilymphoma properties of ethanol extract and subsequent fractions obtained from *Annona cherimola*

	Inhibition of hyperperistalsis IC_{50} (mg/kg)[a]	Antilymphoma activity EC_{50} (mg/kg)[a]
Treatment		CD-1 mice
EELAcR	1.31±0.030[§]	37.5±1.33
AR fraction	0.55±0.010[§]	46.9±1.07
DCM fraction	10.68±0.010	57.7±0.8667
Loperamide-HCl	0.10±0.194	-
Methrotexate	-	0.87±0.15

[a]Data are expressed as mean±SEM (n=6); [§]P<0.05 Correlation coeficient >0.9500. SEM: Standard error of mean; EEL*Ac*: Ethanol extract of the leaves from *Annona cherimola*; DCM: Dichloromethane; AR: Aqueous residual; EC_{50}: 50% effective inhibitory concentration; IC_{50}: 50% inhibitory concentration

properties reputed for *A. cherimola* in Mexican traditional medicine may be due to the presence of flavonol glycosides, rutin, nicotiflorin, and narcissin. Flavonoids have been considered the active component of several antidiarrheic plants.[16-18] On the other hand, the result of the antilymphoma activity demonstrated to the EEL*Ac* is in agree with the antitumor activity previously described to the leaves of *A. cherimola*.[19] The antitumor activity can be associated with the presence of rutin; it is a flavonol glycoside with significant antilymphoma and antileukemia properties. Also, inhibited the skin carcinogenesis and colonic neoplasia as well is attenuated effects caused by cisplatin.[14]

CONCLUSION

Our work confirms the value of the leaves of *A. cherimola* as an important source of antipropulsive and antitumor compounds.[7,11,19] In addition, it can be suggested that flavonoids identified in specific rutin may play an important role in the biological properties of *A. cherimola.* Finnaly, considering that EEL*Ac* and AR fraction exhibited stronger effects in both assays, these results open the possibility of investigating the use of EEL*Ac* and AR fraction as a therapeutic source in the treatment of diarrhea and NHL.

Financial support and sponsorship

We thank IMSS for the contract grant sponsor: FIS/IMSS/PROT/G17-2/1722 and FIS/IMSS/PROT/G18/1807.

Conflicts of interest

There are no conflicts of interest.

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