Revista Brasileira de Farmacognosia 26 (2016) 44–49

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.journals.elsevier.com/revista-brasileira-de-farmacognosia

Original Article

Phytochemical study and evaluation of cytotoxic and genotoxic properties of extracts from *Clusia latipes* leaves

Natalia Bailón-Moscoso[a,b], Juan C. Romero-Benavides[c,d], Monserrat Sordo[a], Javier Villacís[b], Ronald Silva[d], Luisa Celi[b], Mariano Martínez-Vázquez[c], Patricia Ostrosky-Wegman[a,*]

[a] *Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México, DF, Mexico*
[b] *Departamento de Ciencias de la Salud, Sección de Genética Humana, Microbiología y Bioquímica Clínica, Universidad Técnica Particular de Loja, Loja, Ecuador*
[c] *Instituto de Química, Universidad Nacional Autónoma de México, México, DF, Mexico*
[d] *Departamento de Química, Sección de Química Básica y Aplicada, Universidad Técnica Particular de Loja, Loja, Ecuador*

ARTICLE INFO

Article history:
Received 6 March 2015
Accepted 1 August 2015
Available online 10 December 2015

Keywords:
Anti-cancer phytometabolites
Clusia latipes
Cytotoxicity
Genotoxicity
Comet assay

ABSTRACT

Some species of the *Clusia* genus have been shown to have important biomedical properties, including the ability to inhibit tumor growth *in vitro* and the usefulness for skin care. In this study, we examined the cytotoxic effect of hexane, ethyl acetate and methanol extracts from *Clusia latipes* Planch. & Triana, Clusiaceae, leaves on survival of human prostate cancer cells (PC-3), colon cancer cells (RKO), astrocytoma cells (D-384), and breast cancer cells (MCF-7). The ethyl acetate extract displayed the most substantial cytotoxic effect. However, using a Comet assay, we observed that the hexane extract induced a genotoxic effect (DNA damage) on human lymphocytes in an *in vitro* model. Chromatographic purification of the *C. latipes* hexane extract led to the isolation and identification of friedelin, friedolan-3-ol, and hesperidin as active cytotoxic compounds in hexane extract, while β-amyrine was identified as an active cytotoxic compound in the ethyl acetate extract of *C. latipes*, thereby supporting further studies of the molecular mechanisms underlying the effect of these secondary metabolites on cancer cell survival.

Introduction

The Clusiaceae family, also known as Guttiferae, consists of tropical plants and includes approximately 27 genera and 1090 species (Stevens, 2007). The flora of Southern Ecuador includes a large number of species that belong to the family Clusiaceae. The most representative and important genus is *Clusia*, which is known for its useful biomedical properties. The species of this genus have various biological activities, demonstrating their role as a promising source of active biomedical phytocompounds/phytometabolites that can be used for antimicrobial (Suffredini et al., 2006), anticancer (Díaz-Carballo et al., 2012; Monks et al., 2002), antioxidant (Ferreira et al., 2014), anti-inflammatory, and antihepatotoxic activities. Furthermore, members of the Clusia family have been shown to have an inhibitory effect on human immunodeficiency virus (HIV) (Huerta-Reyes et al., 2004; Balunas and Kinghorn, 2005). The most prominent biomedical applications of these species include the treatment of leprosy and headaches, treatment of warts, and the control of obesity (Hemshekhar et al., 2011). Leaves of *Clusia* sp. are often used to soften the skin and have a potential benefit in skin care (Valadeau et al., 2010). Because this genus presents great variability in terms of biological activities, it presents an interesting source of active secondary metabolites to be used for their potential antitumor activities. In the present study, we performed a chemical composition analysis of the active phytometabolites obtained from organic extracts of the leaves of *Clusia latipes*, as well as an evaluation of the cytotoxic and genotoxic activities of these extracts on human cancer cell lines and human lymphocytes.

Materials and methods

Plant material

A total of 10.6 kg of leaves and stems of *Clusia latipes* Planch. & Triana, Clusiaceae, were collected in Gonzanama-Quilanga

* Corresponding author.
E-mail: ostrosky@biomedicas.unam.mx (P. Ostrosky-Wegman).

http://dx.doi.org/10.1016/j.bjp.2015.08.014
0102-695X/

(0°57′46″ Lat. S, 77°11′46″ Long. O, 2300 m.a.s.l.) region of the Loja Province of Ecuador. A sample specimen (PPN-Cl 002) was deposited and identified in the Herbarium of Departamento de Química of the Universidad Técnica Particular of Loja, Ecuador. Plant material was dried at 30 °C for seven days in dryer trays with air flow, and then was reduced to fine particles by manual grinding to a suitable size.

Preparation of extracts

The dried and ground leaves (1540 g) were macerated at room temperature, with hexane (*C. latipes* Hex), ethyl acetate (*C. latipes* EtOAc), and methanol (*C. latipes* MeOH) sequentially for three days with three liter of each solvent, the procedure was repeated three times. The extract were filtered using filter paper, all extracts were concentrated under reduced pressure, and subsequently stored at room temperature and protected from light until further use. For biological studies, stock solutions (1000 μg/ml) were prepared in dimethylsulfoxide (DMSO) and stored at −20 °C. The aliquots were diluted to obtain the appropriate concentrations before use.

Isolation of secondary metabolites

To separate the components from the *C. latipes* extracts: Hex (39 g), EtOAc (28 g) and MeOH (50 g), and a silica gel-60 F254 chromatography column (CC, Merck) were used. Mixtures of these three solvents were used in polarity up to starting with hexane (100%) to obtain the compound separation. Fractions of 200 ml each were collected using a vacuum pump, the solvent was then removed on a rotary evaporator and the residue was recovered with dichloromethane. Thin layer chromatography (TLC) was performed on each fraction for detection of the compounds. Compounds were visualized by spraying with ceric sulfate solution in acid sulfuric followed by heating on a hot plate. Fractions with a similar profile were pooled and purified by conventional procedures: solvents pair technique and micro columns.

The hexane extract (39 g) was submitted to column chromatography eluted with mixtures of three solvents Hex, EtOAc and MeOH were used in polarity up to starting with hexane (100%) to obtain the compound separation, resulting in 428 fractions of 200 ml each. The eluted fractions were evaluated and pooled according to TLC analysis, affording 108 fractions (F-1 to F-108). F-13 was presented as a yellow amorphous material submitted to recrystallization with methanol, obtaining in the end of the process a white amorphous solid, which was identified as friedelin (**1**). The fraction F-32 eluted with Hex:EtOAc 80:20 (v/v), was present as an amorphous solid, it was recrystallized with methanol, identified as friedolan 3-ol (**2**). The fraction F-72 were fractioned by silica gel-60 F254 chromatography column at 1:10 compound: silica gel and EtOAc:MeOH (80:20) as eluent, resulting a yellow amorphous solid identified as hesperidin (**3**). From the extract using EtOAc (28 g), 309 fractions were obtained. The eluted fractions were evaluated and pooled according to TLC analysis, affording 90 fractions (F-1 to F-90), from fraction F-18, eluted with Hex:EtOAc 90:10 (v/v) a amorphous solid was presented, it was recrystallized with methanol obtaining in the end of the process a white amorphous solid, which was identified as β-amyrine (**4**). From the MeOH extract (50 g), 478 fractions were obtained, the eluted fractions were evaluated and pooled according to TLC analysis, affording 127 fractions (F-1 to F-127), no pure compound was obtained from the this extract.

Characterization and identification of secondary metabolites

Melting points were determined using a Fisher Johns apparatus. The ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, on a Varian 400 MHz-Premium Schelded equipment using tetramethylsilane as an internal reference. $CDCl_3$ and DMSO-d_6 were used as solvents; chemical shifts were expressed in parts per million (ppm) and coupling constants (*J*) were reported in (Hz). Mass spectra (MS) were determined by a gas chromatograph (Agilent Technologies 6890 N) coupled to a mass spectrometer (Agilent Technologies 5973 inert).

Cell culture procedures

Four human cancer cell lines were used: PC-3 (prostate cancer), RKO (colon cancer), D-384 (astrocytoma), and MCF-7 (breast cancer). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% antibiotic-antimitotic solution (100 units/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, Gibco), and 1% L-glutamine (2 mM, Gibco). The cells were incubated at 37 °C, 5% CO_2. Viable cells were counted using the trypan blue exclusion method in a hemocytometer.

Cell viability analysis by MTS assay

The MTS cell viability assay was used to assess the inhibitory effects of the extracts on the survival of human cancer cell lines. A total of $3–5 \times 10^3$ cells/well were seeded into 96-well plates and were allowed to adhere for 24 h. The cells were then treated with 50 μg/ml of whole extract, at final volume of 2 ml. Each concentration/assay was performed three times. DMSO was used as a negative control at a final concentration of 0.1% (v/v) and 1 μM doxorubicin was used as a positive control. The cells were incubated with the treatments for 48 h, after which 20 μl MTS (5 mg/ml, Aqueous One Solution Reagent, GIBCO) was added and further incubated for 4 h at 37 °C. The absorbance was measured at 570 nm. The data obtained with cells treated with DMSO were considered as 100% of viability.

Determination of genotoxicity by Comet assay

Whole peripheral blood samples were obtained from three healthy male donors (age range 20–25 years). Heparinized blood samples (20 μl) were cultured at 37 °C in RPMI-1640 medium (1 ml, Gibco) supplemented with 1% L-glutamine (2 mM), and 1% non-essential amino acids (10 mM, Gibco), and were treated with three different concentrations of *C. latipes* Hex extract (25, 35, 50 μg/ml). DMSO (0.5%) and ethyl methane sulfonate (EMS 0.1 μM, Sigma chemical) were used as negative and positive controls, respectively. The cells were treated for 3 h at 37 °C. Aliquots of 50 μl of each sample were used to determine the percentage of viable cells using double staining with fluorescein diacetate-ethidium bromide (FDA/EtBr), counting a total of 200 cells (live-green, red-dead). A Comet assay was performed, as described by Sordo et al. (2001), with minor modifications. The treated cells were centrifuged and resuspended with 150 μl low melting point agarose gel (LMPA, 1%). Then, 75 μl of the mixture was placed in duplicate onto a slides previously covered with 150 μl of agarose gel and immediately covered with a coverslip to make a microgel on the slide. Slides were placed in an ice-cold steel tray on ice for 1 min to allow the agarose to solidify. The coverslip was removed, and 75 μl of LMP (1%) was layered as before. Slides were immersed in an ice-cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris-base, pH 10). After lysis at 4 °C for 1 h, slides were subjected to horizontal electrophoresis. The DNA was denatured for 20 min in electrophoresis running buffer solution (300 mM NaOH and 1 mM Na_2EDTA, pH >13.0). Electrophoresis was conducted for 20 min at 25 V and 300 mA (0.8 V/cm). All technical steps were conducted using very dim indirect light. After electrophoresis, the slides were