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Original Article

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

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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

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(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

gently removed and alkaline pH was neutralized with 0.4 M Tris, pH 7.5. The slides were dehydrated in two steps with absolute ethanol for 1 min each. Ethidium bromide (25 μl of a 20 mg/ml solution) was added to each slide and a coverslip was placed on the gel. DNA migration was analyzed on a Zeiss Axioskop 2 plus microscope with fluorescence equipment (excitation filter 549 nm, barrier filter 590 nm), and the tail length was measured with a scaled ocular. For the evaluation of DNA migration, 100 cells were scored for each sample at 40× magnification.

Statistical analysis

All data are reported as mean ± SEM of three independent experiments. The statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnet post-test (GraphPad Prism 4). Comet data were analyzed using the Kruskal–Wallis and Dunn's multiple comparison post-test (GraphPad Prism 4). $p < 0.05$ was considered to be statistically significant.

Results

Isolation of secondary metabolites from C. latipes leaves

Three extractions were conducted from the of *C. latipes* leaves: (1) Hex, (2) EtOAc and (3) MeOH, and four secondary metabolites were isolated and characterized. From the Hex extract, 108 fractions were obtained and three compounds were identified. From the fraction F-13 eluted with Hex:EtOAc (95:5), 36 mg of a white amorphous solid was isolated and identified as friedelin (**1**). From the fraction F-32 eluted with Hex:EtOAc (80:20), 22 mg of a solid was isolated and identified as friedolan 3-ol (**2**). From the fraction F-72 eluted with EtOAc:MeOH (80:20), 17 mg of yellow amorphous solid was isolated and identified as hesperidin (**3**). From the extract using EtOAc, 90 fractions were obtained. Only from fraction F-18, eluted with Hex:EtOAc (90:10), 8 mg of a solid was isolated and identified as β-amyrine (**4**). Any pure compound was obtained from the MeOH extract. All the compounds were identified based on physical and spectroscopic data, by comparison with the literature (Quintans et al., 2014; Subhadhirasakul and Pechpongs, 2005; Aghel et al., 2008; Gao et al., 2009).

Characterization and identification of secondary metabolites

Physical and spectroscopic constants from friedelin (**1**): m.p. 235–239 °C. EIMS *m/z* (%): 426 (M+, 38), 411 (12), 341 (7), 302 (16), 273 (31), 205 (27), 69 (100). ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm: 0.66 (s, 2H), 0.78–0.98 (m, 15H), 1.11–1.35 (m, 17H), 1.37–1.58 (m, 8H), 1.66–1.71 (m, 1H), 1.79–2.03 (m, 2H), 2.10–2.38 (m, 3H) 7.19 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm: ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm: 22.3 (C-1), 41.5 (C-2), 213.4 (C-3), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.3 (C-7), 53.1 (C-8), 37.5 (C-9), 59.4 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.2 (C-14), 32.4 (C-15), 36.0 (C-16), 29.9 (C-17), 42.8 (C-18), 35.3 (C-19), 28.1 (C-20), 32.7 (C-21), 39.2 (C-22), 6.9 (C-23), 14.8 (C-24), 17.9 (C-25), 20.4 (C-26), 18.8 (C-27), 32.1 (C-28), 35.0 (C-29), 31.7 (C-30).

Physical and spectroscopic constants from friedolan 3-ol (**2**): m.p. 238–241 °C. EIMS *m/z* (%): 428 (M+, 20), 413 (27), 395 (7), 341 (3), 304 (2), 275 (23), 207 (11), 69 (100). ^{1}H NMR (400 MHz, CDCl3): δ ppm: 3.72 (1H, bd, *J* = 3.1 Hz, H-3), 1.86 (1H, dt, *J* = 10.1, 3.0 Hz, H-2a), 1.70 (1H, dt, *J* = 12.8, 3.1 Hz, H-6a), 1.57 (1H, m, H-2b), 1.16 (3H, s, H-28), 0.99 (3H, s, H-29), 0.97 (6H, s, H-26 and H-27), 0.95 (3H, s, H-30), 0.94 (3H, d, *J* = 7.0 Hz, H-23), 0.93 (3H, s, H-24), 0.84 (3H, s, H-25). ^{13}C NMR (100 MHz, CDCl3): δ ppm: 15.8 (C-1), 35.1 (C-2), 72.3 (C-3), 49.2 (C-4), 37.1 (C-5), 41.7 (C-6), 17.9 (C-7), 53.3 (C-8), 37.8 (C-9), 60.2 (C-10), 35.2 (C-11), 30.6 (C-12), 39.4 (C-13), 39.8 (C-14), 32.9 (C-15), 36.1 (C-16), 30.0 (C-17), 42.9 (C-18), 36.2 (C-19), 28.3 (C-20), 32.1 (C-21), 39.3 (C-22), 11.6 (C-23), 16.4 (C-24), 18.2 (C-25), 18.8 (C-26), 20.1 (C-27), 32.3 (C-28), 35.0 (C-29), 31.9 (C-30).

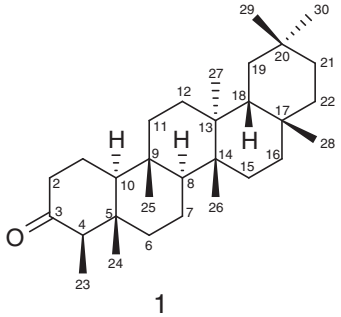
Physical and spectroscopic constants from hesperidin (**3**): m.p. 258–259 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.08 (3H, d, *J* = 6.0, H-6‴), 2.77 (1H, dd, *J* = 3.11, 15.11, H-3eq), 3.07 (1H, dd, *J* = 13.8, 12.0, H-3ax), 3.09–3.86 (2H, m, H-2″-H-5‴), 3.77 (3H, s, 4′-OCH_3), 4.52 (1H, d, *J* = 7.8, H-1″), 4.97 (1H, s, H-1‴), 5.49 (1H, dd, *J* = 3.11, 15.11, H-2), 6.12 (1H, sl, H-8), 6.15 (1H, sl, H-6), 6.92 (3H, m, H-2′, H-5′, H-6′), 12.06 (1H, s, 5-OH), ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 78.4 (C-2), 42.1 (C-3), 197.0 (C-4), 163.1 (C-5), 96.4 (C-6), 165.2 (C-7), 95.6 (C-8), 162.5 (C-9), 103.3 (C-10), 130.9 (C-1′), 114.2 (C-2′), 146.5 (C-3′), 147.9 (C-4′), 112.0 (C-5′), 117.9 (C-6′), 100.6 (C-1″), 73.0 (C-2″), 76.3 (C-3″), 69.6 (C-4″), 75.5 (C-5″), 66.1 (C-6″), 99.5 (C-1‴), 70.3 (C-2‴), 70.7 (C-3‴), 72.1 (C-4‴), 68.3 (C-5‴), 17.8 (C-6‴), 55.7 (4′-OCH_3).

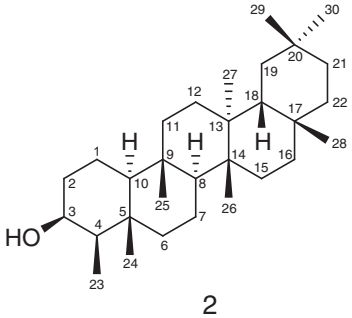
Physical and spectroscopic constants from β-amyrine (**4**) m.p: 238–241 °C. EIMS *m/z* (%): 426 (M+, 51), 411 (20), 341 (10), 287 (13), 273 (61), 246 (33), 220 (18), 205 (57), 161 (29), 125 (85), 95 (100), 69 (97), 55 (56), 41 (30). ^{1}H NMR (400 MHz, CDCl3): δ ppm: 0.68 (1H, d, *J* = 11.0), 0.73 (1H, s), 0.77 (1H, s), 0.80 (1H, s), 0.87 (1H, s), 0.90 (1H, s), 0.93 (1H, s), 1.07 (1H, s), 1.19 (1H, s), 1.70 (1H, td, *J* = 4.3, 13.5 Hß), 1.8 (1H, m), 1.89 (1H, td, *J* = 4.0, 14.0 Hß), 1.93 (1H, dd, *J* = 4.0, 13.7 Hß), 3.15 (1H, dd, *J* = 4.4, 10.8 Hz, H-3), 5.12 (1H, t, *J* = 3.2). ^{13}C NMR (100 MHz, CDCl3): δ ppm: 38.7 (C-1), 27.2 (C-2), 79.3 (C-3), 38.5 (C-4), 55.1 (C-5), 18.6 (C-6), 32.4 (C-7), 39.8 (C-8), 47.6 (C-9), 36.9 (C-10), 23.6 (C-11), 121.7 (C-12), 145.2 (C-13), 41.7 (C-14), 26.2 (C-15), 26.1 (C-16), 32.6 (C-17), 47.2 (C-18), 46.8 (C-19), 31.0 (C-20), 34.6 (C-21), 37.2 (C-22), 28.1 (C-23), 15.3 (C-24), 15.3 (C-25), 16.7 (C-26), 25.7 (C-27), 28.5 (C-28), 33.8 (C-29), 23.7 (C-30).

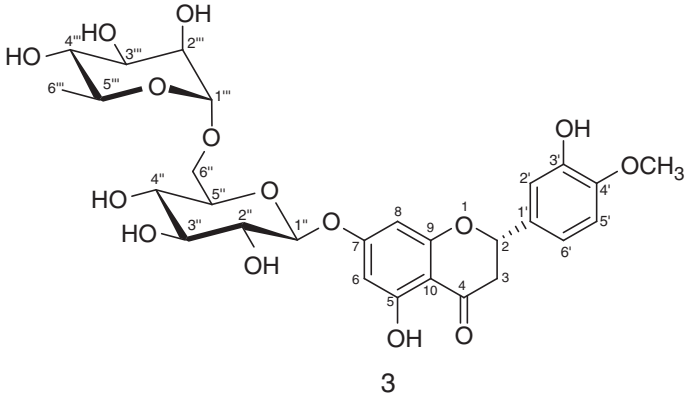
Table 1
Effect of *Clusia latipes* extracts in the growth of human cancer cell lines.

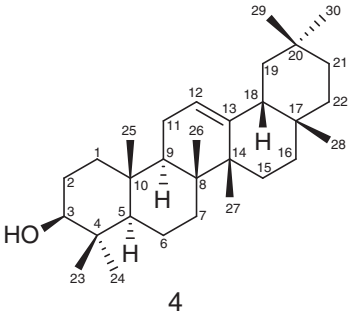
Treatment (50 μg/ml)	% of inhibition ± SEM[a] Human cancer cell lines			
	D384 (Astrocytoma)	PC-3 (Prostate cancer)	MCF-7 (Breast cancer)	RKO (Colon cancer)
Clusia latipes Hex	29.3 ± 4.8	1.8 ± 4.7	27.2 ± 5.8	25.4 ± 6.2
Clusia latipes AcOEt	33.5 ± 2.7	44.6 ± 3.8	33.7 ± 3.6	31.9 ± 6.4
Clusia latipes MeOH	24.7 ± 4.0	41.2 ± 3.4	9.5 ± 3.7	19.3 ± 4.1
Doxorubicin 2 μM	77.8 ± 1.5	70.9 ± 3.3	75.9 ± 2.1	88.2 ± 1.3

[a] Mean and standard error (SEM) of at least three independent experiments. Control cells were considered as 100% viability.









Cytotoxic effect of whole extracts on human cancer cell lines

The effect of secondary metabolites on cell viability was determined by an MTS assay using human cancer cell lines treated for 48 h with whole extracts of *C. latipes* (50 μg/ml). Nearly all of the tested extracts inhibited the viability of the cancer cell lines by up to 25% (Table 1). However, the Hex extract was unable to inhibit the viability of the PC-3 prostate cancer cell line (1.8%), and the MeOH extract showed a low inhibitory effect on MCF-7 cells (~9.5%), while it decreased the viability of the PC-3 prostate cancer cells by ~40%. The EtOAc extract was shown to be the most active in decreasing viability of all cancer cell lines (by more than 30%), and was more effective on the PC-3 prostate cancer cell line (~44%).

Genotoxic effect of Hex extract in human lymphocytes

In the present study, the Hex extract was selected to evaluate genotoxicity because it has molecules with protective potential, such as hesperidin (antitumor) and friedelin (anti-inflammatory) (Tanaka et al., 1997; Ahmadi et al., 2008; Hosseinimehr et al., 2009; Antonisamy et al., 2011; Sahu et al., 2013). Human lymphocytes were used for the experiment. We examined the possible cytotoxic and genotoxic effects of *C. latipes* Hex extract using double fluorescein diacetate/ethidium bromide staining (FDA/EtBr) and the Comet assay, respectively. The viability of the cells after exposure to the Hex extract (25, 35, and 50 μg/ml) was greater than 70% (Fig. 1A), indicating that those doses were not cytotoxic. The positive control treated with EMS caused significant ($p < 0.001$). A clear and statistically significant increase in DNA migration was found in cells exposed to extracts compared ($p < 0.001$) tail length (Fig. 1B). The genotoxic effects determined by the Comet assay displayed a dose-dependent response.

Discussion

The evaluation of toxicity or protective effects of various species used in traditional medicine is essential, and is increasingly receiving attention for their possible application in diseases such as cancer (Moreira et al., 2014). This study reports the chemical composition of various organic extracts obtained from *C. latipes* leaves and their *in vitro* antitumor activities (cytotoxic and genotoxic). The presence of beta-amyrin, friedelin and friedelan-3-ol is consistent with compounds commonly isolated from other species of the same genus (Reyes-Chilpa et al., 2004; Teixeira et al., 2006; Mangas Marín et al., 2008; Ribeiro et al., 2011). However, in the present study, we successfully isolated and identified the presence of hesperidin in *C. latipes*, which represents the first report of this molecule from the genus *Clusia*. The presence of hesperidin may justify the use of *C. latipes* in traditional medicine and skin care, because this flavone has been shown to inhibit lesions of atopic dermatitis and the elevation of IgE in plasma of NC/Nga mice (Nagashio et al., 2013), as well as to stimulate epidermal proliferation in normal mouse skin (Hou et al., 2012). However, hesperedin also has anti-proliferative effects in tumor cells, including breast cancer (Natarajan et al., 2011) and colon cancer (Park et al., 2008). In addition, the hesperidin displayed cytotoxic effects on cell lines that show drug resistance, which was related to the ABC transporter function (Bailón-Moscoso et al., 2014). In the present study, the observed anti-proliferative effect of the extracts on selected cancer cells could be attributed to the presence of hesperidin, as well as the two triterpenes isolated, friedelin (**1**) and friedelan-3-ol (**2**). In similar studies, it has been observed that both friedelin and friedelan-3-ol have cytotoxic activity in other cell lines (Hela and CEM-SS), with IC_{50} values between 3.5 μg/ml and 11.8 μg/ml (Utami et al., 2013).

Contrary to expectations, the *C. latipes* Hex extract induced a significant increase ($p < 0.001$) in DNA damage in lymphocytes exposed to the concentrations used in this study. Because the potential chemical or metabolic interactions of the phytometabolites from the extracts with the oxidative cell machinery may produce reactive oxygen species, we suggest that the tested phytometabolites could lead to the formation of free radicals. These free radicals are capable of inducing DNA strand breaks, which were

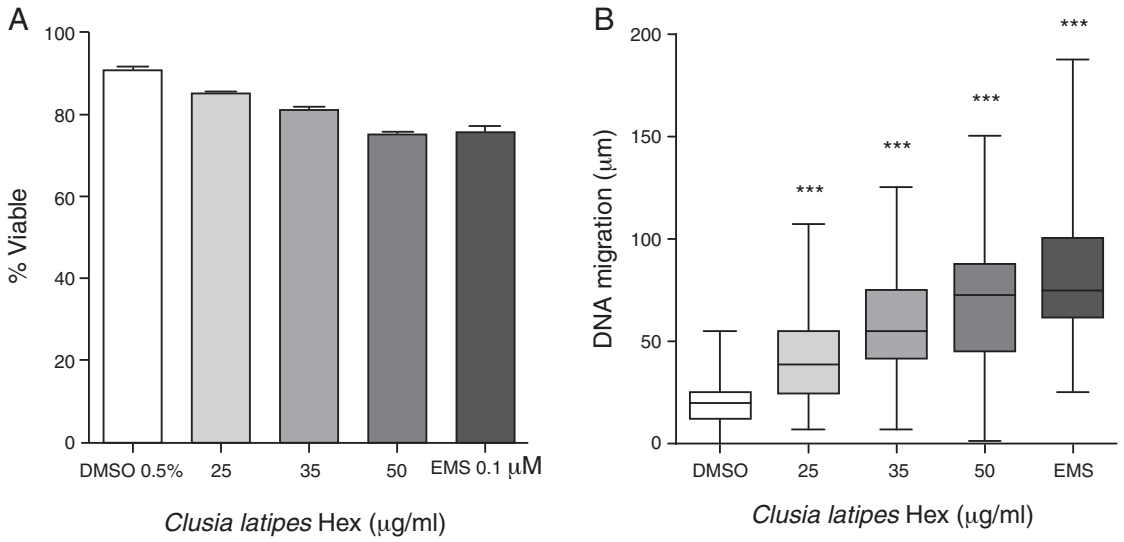


Fig. 1. Cytoxicity and genotoxicity effects of *Clusia latipes* Hex extract on human lymphocytes exposed to 25, 35, and 50 μg/ml. (A) Cell viability determined by FDA/EtBr. (B) DNA damage determined by Comet assay. EMS was used as a positive control. ***$p < 0.0001$ with respect to control group (Kruskal–Wallis and Dunn's tests).

detected by the Comet assay. The damage detected by this method is repairable DNA damage; thus, it is necessary to perform other experiments to determine the genotoxic effects.

Further studies are needed to corroborate the cytotoxic and genotoxic effects of this mixture and to provide a molecular mechanistic foundation of these effects prior to any potential antineoplastic use.

Authors' contributions

NBM (Ph.D. student) contributed by collecting plant samples, conducting the laboratory work, analyzing the data and drafting the paper. MS, JV and LC contributed to the biological studies. JCRB and RS contributed by obtaining extracts and isolating and characterizing secondary metabolites. MMV contributed by critical reading of the manuscript. POW designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All of the authors read the final manuscript and approved the submission.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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