Antiproliferative Activity of Haematoxylum brasiletto H. Karst

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ABSTRACT
Background: Haematoxylum brasiletto is a tree that grows in Central America, commonly known as “Palo de Brasil,” which is used in the traditional medicine for the treatment of cancer and gastric ulcers. Objective: The aim of this study was to isolate the compounds responsible for antiproliferative activity of H. brasiletto. Materials and Methods: A bioassay-guided fractionation of ethanol extract of H. brasiletto was performed using 3-I, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide cell proliferation assay to measure the antiproliferative activity on six human cancer cell lines (A549, LS180, HeLa, SiHa, MDA-MB-231, and NC-I-H1299) and one human noncancer cell line (ARPE-19). The ethanol extract was partitioned with hexane, dichloromethane, and ethyl acetate. The active dichloromethane fraction was fractioned by silica-column chromatography, and active subfractions were separated using preparative-thin layer chromatography. The chemical structure of an isolated compound was elucidated with different chemical and spectroscopic methods. Results: The flavonoid brazilin (1) was isolated from the heartwood of H. brasiletto. The measurement of antiproliferative activity showed that brazilin can inhibit the growth of SiHa, MDA-MB-231, A549, and NCI-H1299 cancer cell lines by 50% at doses of 44.3, 48.7, 45.4, and 48.7 μM, respectively. Furthermore, the flavonoid showed a high antiproliferative activity on LS 180 and HeLa with IC50 values of 62.2 and 71.9 μM, respectively. Brazilin also exhibited a high antiproliferative activity on the human noncancer cell line ARPE-19 with an IC50 value of 37.9 μM. Conclusions: Brazilin is able to inhibit the growth of SiHa, MDA-MB-231, A549 and NCI-H1299 cancerous cell lines. Brazilin exhibited a moderate antiproliferative activity on the human non-cancer cell line ARPE-19. Brazilin demonstrated to have antiproliferative activity against human cancer cell lines and could be a potential source of anticancer agents.

Key words: Antiproliferative activity, flavonoids, Haematoxylum brasiletto

SUMMARY
• The flavonoid brazilin was isolated from the heartwood of H. brasiletto
• Brazilin is able to inhibit the growth of SiHa, MDA-MB-231, A549 and NCI-H1299 cancerous cell lines
• Brazilin exhibited a moderate antiproliferative activity on the human non-cancer cell line ARPE-19
• Brazilin demonstrated to have antiproliferative activity against human cancer cell lines and could be a potential source of anticancer agents.

INTRODUCTION
Cancer is a group of diseases that represent a serious public health problem.1 Cancer is the second highest cause of morbidity and mortality worldwide, with approximately 14.1 million new cases and 8.2 million cancer-related deaths. In addition, 32.6 million people are currently living with some kind of cancer.2 Thus, cancer is one of the main causes of death in developed countries.3 Some of the most common cancer types, such as breast, cervical, oral, and colorectal cancers have high cure rates when detected early and treated according to effective means, including surgery, radiation therapy, and chemotherapy. However, all these treatments are also accompanied by severe side effects such as tingling, burning, weakness or numbness in the hands, feet, or both, weak, sore, tired, or achy muscles, loss of balance, and shaking or trembling.4

The use of natural products derived from plants, animals, or microorganisms for medicinal purposes has a long history.5 Medicinal plants constitute an important natural source of bioactive compounds with multiple applications that can be used for therapeutic purposes. Their chemical components, with possibly novel mechanisms of action, provide the basis for the synthesis of pharmaceutical products.6 Plants are the major source of anticancer drugs.7 In this way, ethnobotanical

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knowledge of medicinal plants represents an alternative for the identification of secondary metabolites with antiproliferative activity that could be a promising source of future anticancer drugs.[8,9]

Haematoxyllum brasiletto H. Karst (Fabaceae), native from Mexico and extending into Central America, commonly known as “Palo de Brasil,” is used by rural communities in the State of Guerrero, Mexico, as traditional treatment for hypertension, stomach upsets, mouth infections, diarrhea, gastric ulcers, and cancer.[10] An ethanolic extract of the stem bark of H. brasiletto was found to inhibit the growth of Escherichia coli O157:H7 (EHEC), verotoxin production, and adhesion of E. coli O157:H7 to HeLa cells.[11] H. brasiletto has shown antimicrobial activities against Staphylococcus aureus 375, S. aureus ATCC 25923, and Enterococcus fæcium 379.[12]

To provide scientific validation of traditional medicinal use of H. brasiletto for the treatment of cancer, in the present study, We evaluated the bioguided antiproliferative activity of H. brasiletto.

MATERIALS AND METHODS

Chemicals and reagents

All solvents used were of analytical grade. Methanol (PubChem CID: 887), ethanol (PubChem CID: 702), n-hexane (PubChem CID: 8058), dichloromethane (PubChem CID: 6344), ethyl acetate (PubChem CID: 8857), and sulfuric acid (PubChem CID: 1118) were purchased from Fermont chemicals (Monterrey, NL, Mexico). CD OD was purchased from Cambridge Isotopes Laboratories, Inc., (Tewksbury, MA, USA). Water was purified by Milli-Q instrument (Millipore, Bedford, MA, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) high-glucose, L-glutamine solution 200 mM (PubChem CID: 24895310), L-arginine monohydrochloride (PubChem CID: 87640969), L-asparagine (PubChem CID: 24890831), sodium pyruvate solution 100 mM (PubChem CID: 24899804), penicillin–streptomycin solution (PubChem CID: 86591708), doxorubicin hydrochloride (PubChem CID: 31703), dimethyl sulfoxide (DMSO) (PubChem CID: 679), trypsin–EDTA solution 0.25%, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (PubChem CID: 64965), phosphomolybdic acid (PubChem CID: 24845315), and ceric sulfate (PubChem CID 159684) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was obtained from Gibco Life Technologies (Grand Island, NY, USA). Silica gel 60 (70–230 mesh) and silica gel 200–400 mesh (0.2 mm thickness) were obtained from E.M. Merck (Darmstadt, Germany). Bovine serum was obtained from Gibco Life Technologies (Grand Island, NY, USA).

Plant material

The heartwood of H. brasiletto was collected at Mochilán, Guerrero, Mexico. 99°21′19.03″ W; 17°29′03.27″ N to 1042 mm snm in March 2015. The specimens were taxonomically identified by Professor Maria de las Angeles Venalonzo Martínez, a voucher of classification was assigned (UAGROHHB115) and was deposited in the Herbarium of Universidad Autónoma de Guerrero. All plant materials were air-dried in the shade at room temperature. The dried samples were powdered and stored at 4°C.

Preparation of ethanolic extract and solvent fractions

The plant extract was obtained based on the methodology described by González-Salvatiera et al.[11] In brief, the ethanolic extract of the powder of heartwood of H. brasiletto (1 kg) was obtained by maceration with 96% ethanol (EtOH) at room temperature for 10 days with regular manual stirring twice daily. The combined EtOH extracts were evaporated under reduced pressure in a rotatory evaporator to yield the crude extract (50 g). The crude extract (HBM-1) was suspended in 250 ml of an aqueous (3:2 water [H₂O]/methanol [MeOH]) mixture and the resulting suspension was fractioned by successive liquid–liquid partition with n-hexane (Hx), dichloromethane (CH₂Cl₂), and ethyl acetate (EtOAc) to produce the corresponding low (HBM-2A [500 mg]), low-medium (HBM-2B [5 g]), and medium (HBM-2C [20 g]) polarity fractions, respectively. The ethanol crude extracts and their fractions were analyzed by TLC. A sample of 100 μg is dissolved in 100 μL of CH₂Cl₂ and is applied to the plate using a capillary tube, and then placed in a chromatographic chamber and eluted with a suitable system. The plate is observed under ultraviolet (UV) light and the bands of interest were evaluated for their retention factor (Rf). Chromatographic analyses were performed on 5.5 cm², 0.2 mm thick silica gel plates (E.M. Merck DC Alufolien). All extracts were stored at −4°C in amber glass vials until use.

Isolation of bioactive metabolites of Haematoxyllum brasiletto

Column chromatography (CC) was performed using silica gel 60 (70–230 mesh, Sigma). While silica gel 200–400 mesh (Sigma) was used for flash CC, vacuum liquid chromatography (VLC) purifications were carried out using TLC-grade silica gel (Merck). Gel-preparative TLC purifications were performed using glass-backed 20 cm × 20 cm silica gel plates (2.0 mm thickness, Merck). For analytical TLC analyses, aluminum-backed silica gel plates (E.M. Merck, 0.2 mm thickness) were used. Chromatograms were examined under UV light and then visualized by dipping the plates in a solution of phosphomolybdic acid (20 g) and ceric sulfate (2.5 g) in 500 mL of sulfuric acid (5%), followed by drying and heating to 100°C.[14]

VLC purification of the bioactive dichloromethane fraction (5 g), using a gradient elution with mixtures of CH₂Cl₂:EtOAc: MeOH, produced 11 major fractions (3A-3K). Fraction 3B (1.45 g) was purified by CC elution with n-hexane: EtOAc: MeOH (45:50:5) to produce 8 new fractions (4A-4H). Final purification of fraction 4C (152.5 mg) using a multiple elution (3x) preparative-TLC eluting with EtOAc: CHCl₃:MeOH (70:30:10 + 50 μL of formic acid/10 mL of solution) resulted in the isolation of brazilin (1) (54 mg), which was identified by comparing its spectroscopic and spectrometric data with those in literature.[13]

Structure elucidation

The structure of isolated compound was determined by different spectroscopic analyses such as IR, NMR spectra (¹H NMR, ¹³C DEPT 135 and DEPT 90), and two-dimensional experiments, such as hydrogen–hydrogen correlation (H–H COSY), heteronuclear multiple bond coherence (HMBC), heteronuclear single quantum coherence (HSQC), mass spectrometry, and also by comparison with literature data. For obtaining IR spectrum, we used a Bruker Tensor 27 spectrometer coupled with ATR. ¹H NMR and ¹³C NMR were acquired on a Bruker Avance III, operating in 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR. Samples were dissolved in CDOD. Chemical shifts were given in δ (ppm), and coupling constants (J) are expressed in Hertz (Hz). Residual not deuterated solvent peak (δH 4.87, 3.31 and δC 49.15) was set as reference, and tetramethylsilane (TMS) was used as an internal standard. DART-MS (Direct Analysis in Real Time Mass Spectrometry) was measured using a Joel AccuTOF JMS-T100 LC Mass Spectrometer (Japan) and positive ion [M+ H⁺]⁺ was identified. Fourier-transform infrared (FT-IR) spectra were taken on a Bruker Tensor 27 spectrometer with photodiode detector using KBr pellets method for sample preparation.
Cell lines and cell culture

Cell lines such as ARPE-19 (human retinal pigmented epithelium), HeLa (human cervix carcinoma), SiHa (human cervix squamous cell carcinoma), MDA-MB-231 (human mammary gland epithelial adenocarcinoma), NCI-H1299 (human lung carcinoma; nonsmall cancer cell), A549 (human alveolar adenocarcinoma), and LS 180 (human colorectal adenocarcinoma) were purchased from the American Type Culture Collection (ATCC, Rockville, MD).[16] All cell cultures were maintained in DMEM supplemented with 5% heat-inactivated fetal calf serum (D5F) and grown at 37°C at an atmosphere of 5% CO2.

Cell proliferation assay

To evaluate the effect of plant extracts on the proliferation of seven cell lines, cell proliferation was determined using the standard MTT assay.[17] In brief, 10,000 cells (50 µL) were added into each well of a flat 96-well plate. After 12 h incubation at 37°C at an atmosphere of 5% CO2, to allow cell attachment, the cell cultures were incubated with 50 µL of medium containing different concentrations of either crude extract or fractions, and the cell cultures were incubated for 48 h. The crude extract or fraction was first dissolved in DMSO and then diluted in D5F. Control cell cultures were incubated with DMSO (final concentrations of DMSO: 0.06%–0.5%). The antitumor drug doxorubicin was used as a positive control due to its wide use in the clinic for the treatment of a broad spectrum of cancers.[18]

In the last 4 h of the cell culture, 10 µL of MTT stock solution (5 mg/mL) was added to each well. Formazan crystals were dissolved with acidic isopropanol, and the plates were read in an ELISA plate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbance of the wells was read within 15 min of adding isopropanol. The antiproliferative activity of extracts was reported as IC50 values (IC50 was defined as the concentration of extract evaluated which inhibits cell proliferation by 50%).[19] Values of proliferation of at least three experiments, in triplicate, were log transformed, normalized, and nonlinear regression analysis was used to generate a dose-response curve to calculate IC50 values. The differences in means were analyzed using one-way analysis of variance (one-way ANOVA) followed by Tukey’s test GraphPad Prism 5 (GraphPad Software, Inc., CA, USA).

RESULTS

Flavonoid isolated from ethanol extract of Haematoxylum brasiletto

In this study, the in vitro antiproliferative activity of the ethanol extract and isolated compound from H. brasiletto against seven cell lines was evaluated. Purification of the dichloromethane partition of ethanol extract of the heartwood of H. brasiletto led to the isolation of one known compound: brazin, (C16H12O8) (6α,11βR)-7,11b-Dihydro-6H-indeno[2,1-c] cromeno-3,6a,9,10-tetrol [Figure 1]. brazin was characterized and identified by its spectroscopic data (1H NMR, 13C NMR, DEPT, COSY HSQC, HMBC, and IR) and by comparison with published values [Table 1], this compound was previously described.[10] The flavonoid brazin has been previously isolated from H. brasiletto[10,21] and Caesalpinia sappan.[21]

Antiproliferative activity

Recently, the antiproliferative activity of the extracts of H. brasiletto against A549, RAW 264, and L-929 cells was evaluated using the MTT assay, which demonstrates mitochondrial activity of cells and is commonly used to measure the cell viability. These previous results prompted us to perform the present study in which the aim was to isolate the compounds responsible for antiproliferative activity of the ethanol extract of H. brasiletto.

The flavonoid isolated of chromatographic fraction of dichloromethane of H. brasiletto was evaluated for its effects on proliferation of a panel of six human cancer cell lines (A549, LS180, H1299, HeLa, SiHa, and MDA-MB-231) and a normal (noncancer) human cell line (ARPE-19). The broad-spectrum chemotherapeutic agent, doxorubicin, was included as a positive control and for comparison purposes as it can induce apoptosis for intercalation into DNA and disruption of topoisomerase-II DNA repair.[22] The results are presented in Table 2. The antiproliferative activity of brazin was evaluated [Table 2], demonstrating that it causes a moderate inhibitory effect on the growth in the human SiHa, MDA, A549, and H1299 cell lines at IC50 values of 44.3, 48.7, 45.4, and 48.7 µM, respectively. In LS180 and HeLa, a low effect at IC50 62.2 and 71.9 µM, respectively, was observed. Brazin showed a moderate antiproliferative effect on noncancer ARPE-19 cell line with IC50 value of 37.9 µM, suggesting that the antiproliferative activity of brazin is nonselective.
There are no conflicts of interest.

Nil.

1b-Dihydro-6 with antiproliferative activity from In this study, using a bioassay-guided method, we isolated one flavonoid IC with antiproliferative activity of flavonoid brazilin from In the same way, kaempferitrin, isolated from xanthimicrol, exhibit an IC values >100 µM in HeLa. Likewise, other natural products isolated from Sonora propolis in Mexico, such as CAPE, galangin, and has IC values > 45 µM in A549 cells. In the same way, kaempferitin, isolated from Justicea spicigera, shows IC values >45 µM in HeLa. In this study, using a bioassay-guided method, we isolated one flavonoid with antiproliferative activity from H. brasiletto: brazilin (6aS,11bR)-7,1 1b-Dihidro-6H-indeno[2,1-c]cromeno-3,6a,9,10-tetrol; this compound demonstrated antiproliferative activity against selected human cancer cells.

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Conflicts of interest There are no conflicts of interest.

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