SUPPLEMENTARY MATERIAL

Genotoxicity, Cytotoxicity and Chemical profile from Inga laurina (FABACEAE)

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ABSTRACT

This study aimed to evaluate the cytotoxicity and genotoxicity from Inga laurina leaves extracts and fractions and obtain their chemical profile. The chemical profile of the crude extract from I. laurina leaves and its fractions was investigated through $^1$H NMR, RP-HPLC-PDA by co-injection with authentic standards and HPLC-MS. The quinone reductase induction as a biomarker for cancer chemoprevention was evaluated in murine hepatocellular carcinoma line, whereas the cytotoxicity was evaluated by sulforhodamine B assay (SRB) using HepG2 cell line and genotoxicity was evaluated by comet assay. The phytochemical analysis of the leaves crude extract and its fractions showed the presence of 2-hydroxyethyl-dodecanoate and the phenolic compounds: gallic acid, methyl gallate, p-coumaric acid, cinnamic acid, myricetin-3-O-(2"-O-galloyl)\textemdash\textalpha\textemdash rhamnopyranoside, proanthocyanadin A-2 and myricetrin. All the fractions tested were not considered cytotoxic against the selected human cancer cell lines, they did not cause genotoxic in some concentrations damage and induced the enzyme quinone reductase.

KEYWORDS: Inga laurina, Phenolic compounds, Fabaceae, Genotoxicity, Cytotoxicity
**Experimental section**

**General experimental procedure**

NMR spectral data were obtained on a Varian Inova-500 instrument, at 125 MHz for $^{13}$C and 500 MHz for $^1$H. Mass spectral data were obtained on a HPLC-MS/MS 3200 QTRAPR equipment and HPLC-PDA analyzes were performed on a Shimadzu® chromatograph (Shimadzu SPD-M20A). The same chromatographic conditions were used for both HPLC-PDA and HPLC-MS/MS analyzes. Silica gel 60 (230-400 mesh, Merck® KGaA, Germany) was used for chromatographic column and solvents used in the preparation of extracts and fractions were all analytical grade obtained from Vetec®.

**Plant Material**

The species *I. laurina* was collected in Assis (São Paulo State, Brazil) on April 16th, 2008 and identified by Dr. Giselda Durigan. Exsicates (FEA 3552) were deposited in the Botanical Collection of Assis State Forest.

**Plant Extraction, Isolation of compounds and chemical profile**

Leaves (1.0 kg) of *I. laurina* were dried at room temperature, ground and extracted with EtOH using ultrasonic bath (UNIQUE®) for 20 minutes. After evaporation under reduced pressure, the EtOH extract was submitted to liquid-liquid partition with organic solvents, which afforded a hexane fraction (FH, 1.5 g), EtOAc fraction (FAc, 2.6 g), n-butanol fraction (FB, 1.2 g) and hydromethanol fraction (FHa, 3.1 g). In the search for low polarity bioactive compounds, the hexane fraction (FH, 1.5 g) of *I. laurina* was initially selected for investigation and submitted to flash column chromatography over silica-gel 60, eluted with gradient binary mixtures of Hex-EtOAc and EtOAc-MeOH providing seven fractions (A1–A7). TLC preparative analysis eluted at 85:15 Hexane/CH₂Cl₂ of the fraction 12 yielded a subfraction (20 mg) containing **compound 1**. $^1$H NMR analysis of fractions 20 and 23 (eluted in EtOAc) showed a mixture of triterpenes.
The fractions with high phenolic content were submitted to the preliminary evaluation of antioxidant activity by TLC test using β-carotene solution in MeOH (0.02%) for the isolation of phenolic antioxidant compounds.

FAc (2.6 g) was selected to further phytochemical study as it was active in the β-carotene test and possibly contained polyphenols. Its fractionation by column chromatography using C18 silica-gel, eluted in 95:5 H2O/MeOH gradient to 100% MeOH provided 10 fractions (B1 – B10). Their chemical profiles were obtained by HPLC-PDA analysis monitored at 254 nm using a C-18 analytical column "Luna" Phenomenex® and gradient elution 5-100% MeOH (5 μm particles, injection volume: 40 μL, run time 40min, flow 1mL/min). UV spectral analysis and retention times with authentic standards it was possible to isolate and identify the compounds 2, 3 and 4 from fraction 20 and the fraction 21 obtained in 1:1 EtOAc/MeOH were shown to contain flavonoids and other phenolics as major compounds. The HPLC-PDA analysis of fraction 30 (4.7 mg), obtained in 100% MeOH, evidenced a mixture of phenolic compounds and further analysis by LC-UV-MS and led to the identification and isolation of tannin (5). The BuOH fraction (1.2 g) was also submitted to flash column chromatography over C18-silica-gel which afforded 10 fractions (C1–C10). Their HPLC-PDA analysis under the same chromatographic conditions used for FAc allowed the isolation and identification of compound 6 in fraction C5. The hydrometanol fraction (3.1 g) was analyzed under the same chromatographic conditions, which suggested tannin rich mixtures (figure S1).

**2-hydroxyethyl-dodecanoate (1):** H-NMR (500 MHz, CDCl3) δ: 0.8 (t, J = 7 Hz, CH3), 1.2 (m, -CH2), 1.6 (t, J = 8Hz, -CH2), 2.2 (t, J = 8Hz, -CH2), 3.6 (t, -OCH2), 4.1 (t, -OCH2). C-NMR (125 MHz, CDCl3) δ: 14.1 (C12), 22.6 (C11), 29.1-29.6 (C4-C10), 24.9 (C3), 34.2 (C2), 173.8 (C1), 63.3 (C1’), 61.6 (C2’).

**Gallic acid (2):** H-NMR (500 MHz, DMSO-d6) δ: 6.92 (s, 2H).

**Methyl gallate (3):** H-NMR (500 MHz, DMSO-d6) δ: 6.93 (s, 2H), 3.73 (s, 3H).

**Myricetin-3-O-(2''-O-galloyl)-α-rhamnopyranoside (4):** H-NMR (500 MHz, DMSO-d6) δ: 12.60 (s), 6.95 (s, 2H), 6.92 (s, 2H), 6.38 (s), 6.20 (s), 5.50 (d, J=1.5Hz), 5.47 (dd, J=1.5 Hz), 3.8-3.3 (m), 0.94 (d, J=5.5Hz).

**Proanthocyanidin A-2 (5):** H-NMR (500 MHz, DMSO-d6) δ: 7.00 (dd; 8.0 e 2.0, H-14’), 6.82 (d; 8.4, H-13’), 7.15 (d; 2.4, H-10’), 6.11 (s, H-6’), 2.97 (dd; 17.2 e 4.8, H-4β’), 2.78 (dd; 17.2 e 2.4, H-4α’), 4.26 (m, H-3’), 4.95 (br.s, H-2’), 7.04 (dd; 8.0 e 2.0, H-14), 6.83 (d, 8.0, H-13), 7.17 (d; 2.4, H-10), 6.09 (d; 2.8, H-8), 6.02 (d; 2.4, H-6), 4.42 (d; 3.6, H-4), 4.08 (d; 3.2, H-3).

**Myricitrin (6):** H-NMR (500 MHz, DMSO-d6) δ:
12.67 (s; OH), 6.89 (s), 6.37 (d, J = 2 Hz), 6.20 (d, J = 2 Hz), 5.2 (d, J = 1.5 Hz), 4.0 (m), 3.5 (m), 3.3 (m), 3.1 (m) 0.8 (d, J = 6.5).

Figure S1. Compounds identified from the fractions of *Inga laurina* leaves extracts.

An HPLC-PDA methodology was developed to identify the secondary metabolites present in the studied matrices (leaves extract) through co-injection experiments using authentic standard compounds. Initially, the EtOH extract (5 mg) from leaves of *I. laurina* was dissolved in MeOH/H₂O (1:1, v/v), and submitted to solid phase extraction (SPE) to remove interferents as low polarity constituents. HPLC-PDA analyses of the eluate (20 μL) were performed using a RP18 column and MeOH/ H₂O gradient.

During the elution of the chemical components present in the extract, the exploratory gradient of 5-100% MeOH was used to obtain the chromatographic profiles. Phenolic compounds were identified by the retention times and UV spectra obtained by
HPLC-PDA analysis and by comparison with the retention time and UV spectra of authentic standards. The fractions also were analyzed by $^1$H NMR (500 MHz).

**Cell lines Culture**

Hepatocellular carcinoma of the mouse ATCC (American Type Culture Collection) - Hepa 1c1c7 ATCC: CRL-2026 - which was provided by Prof. Dr. John M. Pezzuto (College of Pharmacy, University of Hawaii at Hilo) and human hepatocellular carcinoma (HepG2; ATCC HB-8065) was kindly provided by Dr. Dayse Maria Favero Salvadori (Department of Pathology of the Faculty of Medicine - UNESP, Botucatu Campus).

The Hepa 1c1c7 cell line were cultured in α-MEM culture (Alpha Modification of Eagle's Medium - Sigma®) without ribonucleosides or Deoxyribonucleosides plus streptomycin 0.1 mg/mL and penicillin 100 U/mL, sodium bicarbonate, kanamycin (Sigma®), pH 7.2 and supplemented with 10% fetal bovine serum (Cultilab®). The strain HepG2 cells were cultured in DMEM culture medium (Modified Eagle Medium Dulbecco's – Sigma®) supplemented with penicillin 100 U/mL and streptomycin 0.1 mg/mL sodium bicarbonate, kanamycin (Sigma®), pH 7.2 and supplemented with 10% fetal bovine serum (Cultilab®).

The cells were cultured in bottles maintained at 5% CO$_2$ and temperature of 37°C until the cell monolayers were confluent at 70–80%.

**Cytotoxicity Evaluation**

For the cytotoxicity assay using Sulforhodamine B (SRB), a suspension of HepG2 cell line containing $1.5 \times 10^4$ cells/well was used. Cells were cultured in 96-well plates and after 24 hours of cultivation, the pure compounds were added following a serial 1:3 dilution starting at a concentration of 200 µg/mL.

After 24 hours of treatment, 50 µL of trichloroacetic acid (TCA) 50% was added at low temperature, and the plates were incubated for 1 hour at 4°C, then the TCA solution was removed, and plates were washed with tap water 3 to 4 times. Was then added 50 µL of SRB solution at 0.4% (dilute acetic acid) and the plates were incubated for 20 minutes at room temperature. After removal of the SRB, the plates were washed 3 to 4 times with 1% acetic acid, dried and dissolved dye with 10 mM Tris Base (Sigma®). After 5 minutes incubation at room temperature, the spectrophotometric
reading of absorbance was performed at a wavelength of 570 nm in the plate reader iMark Microplate Reader (Bio-Rad Laboratories®, Hercules, CA, USA).

Tests were performed in three independent experiments, and the percentage of living cells was calculated in relation to the negative control, representing the cytotoxicity of each treatment, as proposed by Zhang et al., 2004: Live cells (%) Test = Absorbance x 100/Absorbance of the Negative Control.

**Quinone Reductase Assay (QR)**

To perform the quinone reductase (QR) assay was necessary to prepare the following reagents: lysing solution with 0.8% digitonin and 2 mM EDTA, pH 7.8 and test solution made of 25 mM Tris-HCl, pH 7.4, 1 mM glucose-6-phosphate, 50 mM of menadione, 30 µM NADPH, 5, µM FAD, 0.07% (w/v) bovine serum albumin, 0.03% (w/v) MTT, 0.01% (v/v) Tween-20 2 units/mL glucose 6-phosphate dehydrogenase. As a positive control we used the β-naphthoflavone inductor (BNF) at a concentration of 0.1 µM (Song et al., 1999). Reagents obtained from Sigma-Aldrich®, St. Louis, MO, USA.

Hepa1c1c7 cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum in 96-well plates for 24 hours, with about 1 x 10^4 cells/mL in each well. They were then exposed to various concentrations following a dilution series of 1: 3 in a concentration of 200 µg/mL of pure compounds obtained from Inga laurina being solubilized in culture medium for 48 hours before the possible enzyme induction occurred. After the treatment, the culture medium was discarded and added 50 µL of lysis solution. The plate was then incubated for 10 minutes at 37 °C. After lysis, we evaluated the QR activity with the addition of 200 µL of assay solution. The plate was incubated for 5 minutes at room temperature, protected from light and the reading was performed. The absorbance was measured using a Microplate Reader iMark reader (Bio-Rad Laboratories®, Hercules, CA, USA) at 595 nm (Prochaska, Santamaria and Talalay, 1992; Simon et al., 2000; Kang and Pezzuto, 2004).

Data were normalized by subtracting the mean absorbance obtained for each concentration the average absorbance of the reagent blank control. The mean standard deviation of each treatment is usually less than 10%. Cell viability was determined in a parallel plate made, stained with crystal violet to check the cell behavior during the experiment. Then, after 48 h, the treatments were removed, and the cells were stained using 100 uL of 0.2% crystal violet in 2% ethanol solution. The dye was discarded, and
the plate was washed in water and dried naturally. For a spectrophotometric 
determination, the crystal violet was solubilized in 200 μL 0.5% sodium dodecyl sulfate 
(SDS) in 50% ethanol and kept on shaker at room temperature for solubilizing the dye 
completely, the measurement was also performed with absorbance at 595 nm using 
plate reader iMark Microplate Reader (Bio-Rad Laboratories®, Hercules, CA, USA) 
(Prochaska, Santamaria and Talalay, 1992)

The potency of enzyme induction QR is expressed in units per g (U/g). Three 
independent experiments were performed, and the results were compared with positive 
control (β-naphthoflavone).

**Comet Assay**

The following solutions were used in the comet assay. Lysing stock solution of 
2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10. Lysis solution prepared at the 
time of use with Triton X-100, plus DMSO and lysing stock solution. EDTA solution 
with 3.7 g in 50 mL of distilled water and NaOH 100 g in 250 mL of distilled water, the 
electrophoresis buffer was done at the time of use with 10 mL EDTA and 60 mL of 
NaOH in 2L distilled water at 4° C, pH 13. Neutralization solution with 0.4 M Tris at 4° 
C, pH 7.5. Solution of PBS (Phosphate Buffered Saline) concentrated 20 times with 180 
g dry NaCl, 27.3 g of anhydrous Na2HPO4, 4.8 g Na2H2PO4·H2O and distilled water, 
PH between 7.2 and 7.6. Normal melting point agarose (Sigma) (1.2 g diluted in 80 mL 
of 1x PBS at 37° C) was used for the test and low melting point agarose or Low melting 
point LMP (Sigma) (0.05 g diluted in 10 mL of 1x PBS at 37° C).

For genotoxicity evaluation the cell line HepG2 (2.5 x 10^5 cells / well) were 
cultured in 24-well plates and a final volume of 500 μL /well. After 24 hours of culture, 
cells were treated with the compounds extracted *Inga laurina* for 24 hours. Culture 
medium was used as negative control and hydrogen peroxide at 0.01M for 5 minutes 
was the positive control. After 24 hours of incubation with the treatments, the culture 
medium was removed, cells were washed, trypsinized and centrifuged at 1500 rpm for 3 
minutes. After centrifugation, the supernatant was removed, and cells were resuspended 
in 200 μL of low melting point agarose at 37° C. This homogenate was spread onto a 
microscope slide precoated with standard agarose (1.5%, m/v), covered with a coverslip 
large (24 x 60 mm) and placed in at 4° C for 5 minutes. Then, the coverslips were 
removed, and slides were immersed overnight at 4° C in freshly prepared, cold lysing
solution (2.5 mol/l NaCl, 100 mmol/l Na$_2$EDTA, 10 mmol/l Tris; pH 10, with 1% v/v Triton X-100 added just before use).

The slides were removed from the lysis solution and subjected to an alkaline electrophoresis (electrophoresis buffer – 1 mmol/l Na$_2$EDTA and 300 mmol/l NaOH, pH > 13) for 20 minutes. The slides were neutralized in 0.2 M Tris buffer (pH 7.5) for 15 min and fixed with 98% ethanol for 5 min. All slides were stained with 30 μl of 10 μg/ml ethidium bromide in distilled water and 100 nucleoids analyzed in a fluorescence microscope (Nikon Eclipse 50i). Images from nucleoids were captured and analyzed using the software CometScore TM version 1.5 (TriTek). The parameter used in this study was the % DNA in tail. (Møller, 2005).

Figures:

Figure S2. % of DNA in tail detect by Comet Assay in HepG2 cells after 24h treatment with diferent concentrations of crude extract, BuOH fraction, AcEt fraction, hexanic fraction and HMet fraction. NC: negative control; VC: vehicle control; PC: positive control (0.01M); *p<0.05, **p<0.01, ***p<0.001 (vs NC).
Tables:

Table S1. Cytotoxicity and Chemical Profile from fractions of *I. laurina* extract.

| Samples             | Cytotoxicity   | Chemical profile                                           |
|---------------------|----------------|------------------------------------------------------------|
| Hexane Fraction     | Not active     | Triterpenes and fatty acids                                 |
| AcOEt Fraction      | Not active     | Flavonoids, phenolic acids and pipecolic acids             |
| BuOH Fraction       | Not active     | phenolic acids, saponins and flavonoids                    |
| Hydrometanic fraction| Not active     | Glycosylated flavonoids, tannin, catechins and polyphenols |

Table S2. Values of Quinone Reductase induction from fractions and crude extract of *I. laurina*.

| Test Samples       | Concentration (µg/mL) |
|--------------------|-----------------------|
|                    | 200 | 66,67 | 22,22 | 7,41 | 2,47 |
| BuOH Fraction      | 1,9 | 2,4   | 2,7   | 2,3  | 2,9  |
| Hydromethanolic Fraction | 2   | 2,7   | 2,6   | 2,2  | 2,4  |
| Hexanic Fraction   | 3,8 | 3,6   | 3,4   | 2,1  | 2,6  |
| AcOEt Fraction     | 2,9 | 2,2   | 2,6   | 2,3  | 2,8  |
| Crude Extract      | 1,8 | 1,8   | 2,3   | 2,2  | 2,1  |
0 – No activity of the QR enzyme; 1 – No Induction activity of the QR enzyme; > 1
Induction activity of the QR enzyme; 2 - Doubled the activity of the QR enzyme.

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