SUPPLEMENTARY MATERIAL

SHORT COMUNICATION

Chemoprevention assessment, Genotoxicity and Cytotoxicity of flavonoids from *Inga laurina* leaves (FABACEAE)

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ABSTRACT

This study aimed to identify and evaluate the cytotoxicity, genotoxicity, antigenotoxicity and chemoprevention assessment of the flavonoids myricetin-3-O-(2''-O-galloyl)-α-rhamnopyranoside and myricetin-3-rhamnoside from *Inga laurina* leaves extracts. The Quinone reductase induction as a biomarker for cancer chemoprevention was evaluated in murine hepatocellular carcinoma, the cytotoxicity was evaluated by sulforhodamine B assay using HepG2 cell line and genotoxicity was evaluated by comet assay. The results demonstrated that the flavonoids did not show cytotoxicity in HepG2 cells. In the chemoprevention evaluations were not able to promote the induction of Quinone Reductase and also no genotoxic effect was observed by the evaluation of the comet assay in none of the concentrations tested. In the antigenotoxicity test, all compounds had a protective effect against damage induced by hydrogen peroxide and were repaired against damage. Although none of the substances were capable of inducing the enzyme Quinone Reductase at the concentrations tested, the antigenotoxicity results showed a powerful chemoprotective action.

KEYWORDS: Flavonoids, *Inga laurina*, Genotoxicity, Cytotoxicity, Chemoprevention

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. RP-HPLC-PDA analyzes were performed on a Shimadzu® chromatograph (Shimadzu SPD-M20A). 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).

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 H_2O/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 **compound 1 (**53 mg**)** from fraction 20 obtained in 1:1 EtOAc/MeOH. The BuOH fraction (1.2 g) was also submitted to flash column chromatography over C18-silica-gel which afforded 10 fractions (C1–C10). Their RP-HPLC-PDA analysis under the same chromatographic conditions used for FAc allowed the isolation and identification of **compound 2 (**45 mg**)** in fraction C5.

Myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside (1): H-NMR (500 MHz, DMSO-d_6) δ: 12.60 (sl), 6.95 (*s*, 2H), 6.92 (*s*, 2H), 6.38 (sl), 6.20 (sl), 5.50 (*d*, J=1.5Hz), 5.47 (*dd*, J=1.5 Hz), 3.8-3.3 (*m*), 0.94 (*d*, J=5.5Hz).

Myricetin-3-rhamnoside (2): H-NMR (500 MHz, DMSO-d_6) 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).

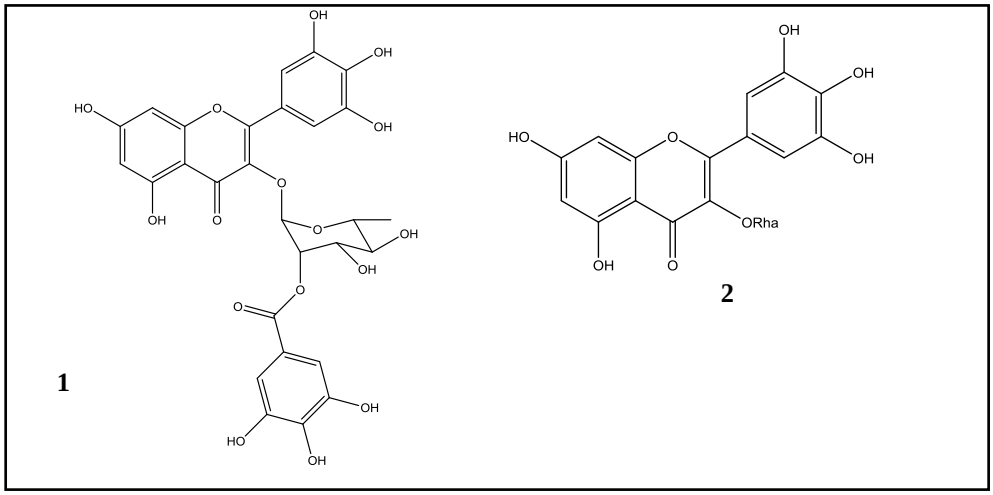

Figure S1. Flavonoids isolated of *Inga laurina* leaves extracts.

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 x 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 40 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 40 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 uL 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 $Na_2H_2PO_4.H_20$ 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 uL/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 uL 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 at 4º 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_2EDTA, 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_2EDTA 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 the tail. (Møller, 2005).

In order to evaluate the antigenotoxicity of flavonoids from *I. laurina*, it was used the hydrogen peroxide (H_2O_2) at concentration of 0.01M in HepG2 cells. *Pre-treatment* and *post-treatment* protocols were used, as proposed by Scolastici et al (2008). These two treatment protocols allowed the identification of the antigenotoxic action at different concentrations of the flavonoids proposed for this study. For the antigenotoxicity assay, HepG2 cells were treated with non-cytotoxic concentrations.

Pre-treatment: The cell suspension of 2.5x105 cells/well was grown in 24 well plates during 24 hours with a final volume of 500 µl/well, then the cells were treated with compounds for 24 hours. As negative control, only culture medium was used and as a positive control the hydrogen peroxide (H_20_2) at 0.01 M for 5 minutes. After 24 hours of incubation with the treatments, 0.01 M hydrogen peroxide (H_20_2) was added for 5 minutes. After the 5 minute period the culture medium was removed, cells were washed, trypsinized, stored in 1.5 ml tubes and centrifuged at 1500 rpm for 3 minutes. After centrifugation, the supernatant was removed and the cells resuspended in 200 µl of agarose (low melting point) at 37 °C. Then,

the volume of each eppendorf was transferred to 2 slides, previously treated with normal melting point agarose. Each blade was covered with a large coverslip (24x60 mm) and placed the refrigerator under the light for 5 minutes. Then the coverslips were removed and the slides were immersed to freshly prepared lysis solution at 4 °C for a minimum of 12 hours under refrigeration. The slides were removed from the lysis solution and subjected to alkaline electrophoresis for 20 minutes. After that, the slides were neutralized and fixed. The slides were analyzed after staining in a fluorescence microscope. 100-nucleoid images were captured and analyzed by TriTek CometScore TM software version 1.5. The parameter adopted in the present study was *% DNA in the tail* (Moller, 2005).

Post-treatment: The cell suspension of 2.5 x 105 cells/well was grown in 24-well plates during 24 hours and at a final volume of 500 µl/well, it was added hydrogen peroxide (H202) to 0.01 M for 5 minutes to induce the formation of cellular damage. After this period the cells were treated with the flavonoids for 24 hours. As negative control, only culture medium was used and as a positive control the hydrogen peroxide (H_20_2) at 0.01 M for 5 minutes. After 24 hours of incubation with the treatments, the culture medium was removed, the cells were washed, trypsinized, stored in 1.5 ml tubes and centrifuged at 1500 rpm for 3 minutes. After centrifugation, the supernatant was removed and the cells re-suspended in 200 µl of agarose (low melting point) at 37 °C. Then, the volume of each eppendorf was transferred to 2 slides, previously coated with normal melting point agarose. Each blade was covered with a large coverslip (24 x 60mm) and placed the refrigerator under the light for 5 minutes. Then the coverslips were removed and the slides were subjected to freshly prepared lysis solution at 4°C for a minimum of 12 hours under refrigeration.

The slides were removed from the lysis solution and subjected to alkaline electrophoresis for 20 minutes. After that, the slides were neutralized and fixed. The slides were analyzed after staining in a fluorescence microscope. 100-nucleoid images were captured and analyzed by TriTek CometScore TM software version 1.5. The parameter adopted in the present study was the *% DNA in the tail.* (Moller, 2005).

Tables

Table S1. ^{1}H and ^{13}C NMR data from **compound 2** (myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside) isolated from *I. laurina* leaves extract.

	myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside		**Myricetin-3-rhamnoside**	
C	**δ_C (ppm)**	**δ_H (ppm)**	**δ_C (ppm)**	**δ_H (ppm)**
2	156.5	-	158.0	-
3	134.3	-	134.2	-
4	177.8	-	178.0	-
5	161.3	12.60 (*s*) - OH	162.0	12.67 (*s*) - OH
6	98.8	6.20 (*s*)	99.2	6.20 (*s*)
7	164.5	-	165.0	-
8	93.6	6.38 (*s*)	94.2	6.37 (*d*, *J* = 2 Hz)
9	156.5	-	157.0	-
10	104.0	-	104.0	-
1’	119.7	-	120.4	-
2’ and 6’	108.0	6.95 (*s*)	108.6	6.89 (*s*)
3’ and 5’	145.8	-	146.4	-
4’	145.7	-	146.3	-
1”	98.8	5.50(*d*, J=1.5Hz)	102.6	5.2 (*d*, *J* = 1,5 Hz)
2”	71.4	5.47 (dd, J=1.5 Hz)	70.7	4.0 (*m*)
3”	70.6	3.8-3.3 (*m*)	71.1	3.5 (*m*)
4”	70.0	3.8-3.3 (*m*)	72.0	3.1 (*m*)
5”	70.5	3.8-3.3 (*m*)	71.4	3.3 (*m*)
6”	17.5	0.94 (*d*, J=5.5Hz)	18.2	0.8 (*d*, *J* = 6.5).
1’”	119.7	-	-	-
2’” and 6’”	108.0	6.92 (*s*)	-	-
3’” and 5’”	145.8	-	-	-
4’”	136.6	-	-	-
C=O	164.5	-	-	-

Table S2. Cell viability from compound 1 (**myricetin-3-rhamnoside**) and compound 2 (**myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside**) isolated from *I. laurina* leaves extract in HepG2 cell line by the sulforhodamine B Assay: Cell viability is given in percentage, from three independent experiments (mean ± standard error -EP) CP: positive control; CN: negative control; One-way ANOVA post-test of Tukey. (***) p <0.001.

Cell viability (%) ± EP		
CP 24.81 ± 2.57***		
CN 58.33 ± 7.78		
Concentration (µg/mL)	**myricetin-3-rhamnoside**	**myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside**
40	58.96 ± 8.14	58.36 ± 6.95
13.3	70.20 ± 10.00	70.92 ± 8.14
4.4	68.81 ± 9.29	70.46 ± 8.84
1.5	69.13 ± 7.96	70.77 ± 9.63
0.5	71.26 ± 8.56	70.36 ± 7.93

Table S3. Values of Quinone Reductase Induction (**RI**) from compound 1 (**myricetin-3-rhamnoside**) and compound 2 (**myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside**) isolated from *I. laurina* leaves extract.

Values of Quinone Reductase induction

Myricetin-3-rhamnoside		
Concentration (µg/mL)	**RI (Treatment/Control)**	**Cell viability (%)**
0.5	0.6 ± 0.28	118.8 ± 11.86
1.5	0.7 ± 0.23	115.0 ± 9.76
4.4	1.1 ± 0.35	112.7 ± 23.8
13.3	1.4 ± 0.94	79.2 ± 8.32
40.0	1.5 ± 0.95	67.4 ± 12.25
β- naphthoflavone	2.0 ± 0.14	119.5 ± 28.23
CB	0.00 ± 0.00	0.00 ± 0.00
CV	0.75 ± 0.00	100 ± 0.00
Myricetin-3-O- (2"-O-galloyl)-α-rhamnopyranoside		
Concentration (µg/mL)	**RI (Treatment/Control)**	**Cell viability (%)**
0.5	0.7 ± 0.29	155.7 ± 37.36

1.5	0.7 ± 0.33	114.6 ± 22.37
4.4	0.6 ± 0.33	110.9 ± 17.17
13.3	0.7 ± 0.24	183.0 ± 86.88
40.0	0.9 ± 0.57	88.7 ± 12.86
β- naphthoflavone	2.0 ± 0.14	119.5 ± 28.23
CB	0.00 ± 0.00	0.00 ± 0.00
CV	0.75 ± 0.00	100 ± 0.00

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.

Figures

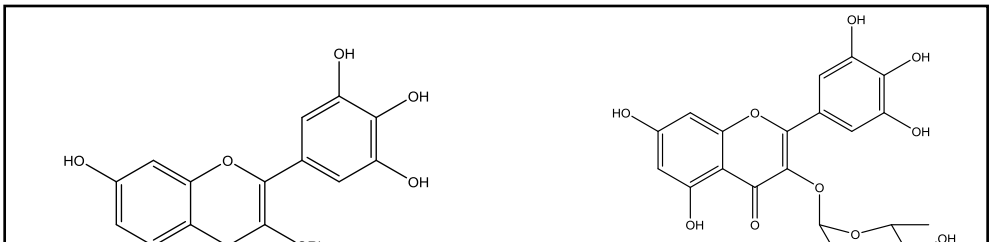

Figure S1. Flavonoids myricetin-3-rhamnoside (**1**) myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside (**2**) isolated from *Inga laurina* leaves extracts.

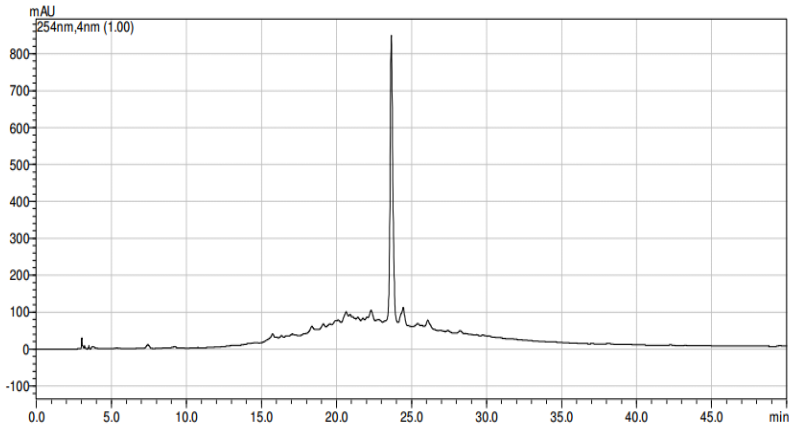

Figure S2. Chromatogram obtained by RP-HPLC-PDA at 254 nm from **compound 2** (myricetin-3-O-(2"-O-galloyl)-α-rhamnopyranoside) isolated from *I. laurina* leaves extract.

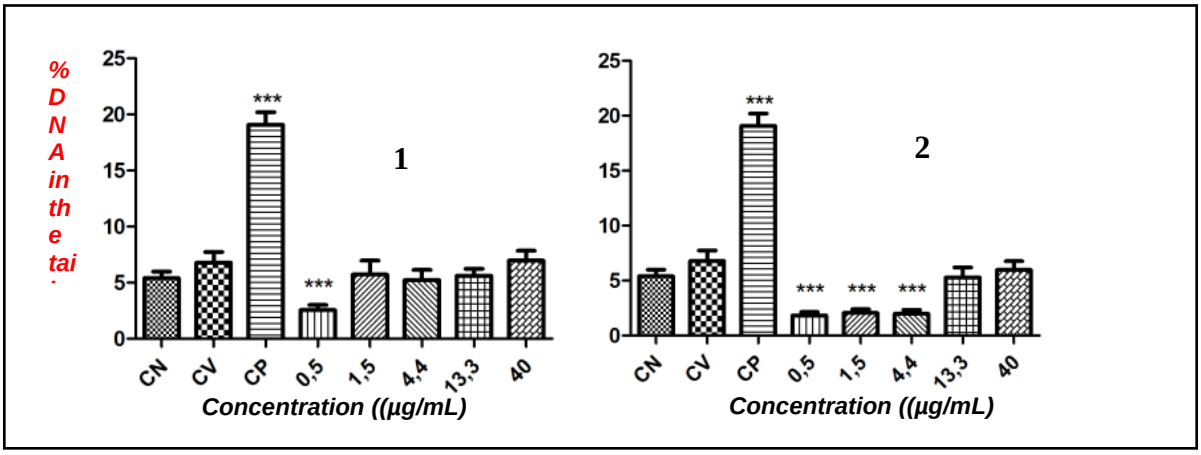

Figure S3. % DNA in the tail detect by Comet Assay in HepG2 cells after 24h treatment at different concentrations of the **compound 1** (myricetin-3-rhamnoside) and **compound 2**

(myricetin-3-O-(2''-O-galloyl)-α-rhamnopyranoside). CN: negative control; CV: vehicle control; CP: positive control (0.01M); *p<0.05, **p<0.01, ***p<0.001 (vs CN).

Figure S4. Antigenotoxicity evaluation in *Pre-treatment* and *Post-treatment* with H_2O_2 at different concentrations of the **compound 1** (myricetin-3-rhamnoside) and **compound 2** (myricetin-3-O-(2''-O-galloyl)-α-rhamnopyranoside). NC: negative control; CP: positive control, CV: vehicle control. *p <0.05, **p <0.01, ***p <0.001 (vs CN).

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