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Original Research Article

Comparative Antioxidant, Antiproliferative and Apoptotic Effects of *Ilex laurina* and *Ilex paraguariensis* on Colon Cancer Cells

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Abstract

Purpose: *To determine and compare the antioxidant, antiproliferative and apoptotic effects of leaf infusions of Ilex laurina and Ilex paraguariensis in colon cancer cells.*
Methods: *Antioxidant activity was determined by ORAC (Oxygen Radical Absorbance Capacity) and FRAP (Ferric Reducing Antioxidant Power). Cytotoxic and antiproliferative effects were analyzed using MTT ((3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) and sulfhorodamine-B respectively. Cell death and apoptosis of human colon adenocarcinoma cells SW480 and their metastatic-derived SW620 cells, were analyzed by flow cytometry using propidium iodide and Annexin-V.*
Results: *Although their flavonoid levels were similar, I. laurina infusion contained 2.2 and 4.4 times higher amounts of total phenolic and caffeoyl derivatives, respectively, than I. paraguariensis. FRAP and ORAC values for I. laurina infusion were 1.6 and 2.0 more active than I. paraguariensis. Both plant infusions inhibited viability and cell growth of SW480 and SW620 cells. These results may be associated to cell cycle-arrest and apoptosis because of the comparable increase of hypodiploid and annexin-V positive colon cancer cells.*
Conclusion: *These data highlight the antioxidant and promising anticancer activities of I. laurina and Ilex paraguariensis.*

Keywords: *Ilex laurina, Ilex paraguariensis, Antioxidant, Antiproliferative, Apoptosis, Colon cancer*

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INTRODUCTION

Colorectal cancer (CRC), a common type of cancer and a major cause of death has been associated with oxidative stress-linked DNA damage [1]. Polyphenols with antioxidant activity have considered an alternative strategy to protect DNA from genotoxicity produced by exposition to reactive oxygen species (ROS) which may occurs during initiation of colon carcinogenesis; additionally polyphenols may interfere in the carcinogenic process by arresting cell cycle and inducing apoptosis of neoplasic cells [2].

Ilex paraguariensis (Aquifoliaceae family), commonly known as yerba mate tea (YMT) is used for preparing a traditional tea-like beverage named mate in Argentina, Southern Brazil, Uruguay and Bolivia. Recently, it has been

reported that mate tea was able to inhibited 50 % of HT-29 and CaCo-2 adenocarcinoma cells growth [3]. These antiproliferative properties have been attributed to some chemical constituents of YMT such as caffeoyl derivatives, quercetin, kaempferol and rutin [4]. Additionally, these compounds confer antioxidant properties of tea mate [5]. A Colombian native plant from this family is the *Ilex laurina* Kunth, which is distributed in the northern central and western mountain ranges, from 1600 to 2900 meters over sea level [6]. Because some species of the genus *Ilex* are closely related, they have been considered subtitutes of *I. paraguariensis* [5]. Thus, to know whether *I. laurina* can be considered an alternative to YMT, as a potential beverage with antioxidant and anticancer properties against CRC, we compared the antioxidant, antiproliferative and apoptotic effects of an infusion obtained from dried leaves of *I. laurina* (Colombia) to a commercial YMT (Argentina) on primary human colon adenocarcinoma cells (SW480) and their metastatic-derived cells (SW620) isolated from a mesenteric lymph node of the same patient [7].

EXPERIMENTAL

Materials

Potassium persulfate (K2S2O8), 6-hydroxy-2,5,7,8-tetramethylchromo-2-carboxylic acid (Trolox®), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), Folin-Ciocalteu reagent, Gallic Acid, Chlorogenic, caffeic, ferulic, p-coumaric acid, Sulforhodamine B (SRB), RNAse A and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). Dulbecco's Modified Eagle Medium (DMEM), horse serum (HS), penicillin-streptomycin solution, insulin-transferrin-selenium-G supplement (ITS), trypsin, non-essential amino acids were obtained from Invitrogen (Cergy-Pontoise, France). Annexin-V FLUOS staining kit from Roche Diagnostics (GmbH, Mannheim, Germany). Other reagents were purchased from Merck (Darmstadt, Germany) and used as received.

Preparation of plant materials and infusions

Leaves of *Ilex laurina* were collected in June 2011 in the village of Santa Helena, municipality of Medellín (Antioquia, Colombia) and identified by Dr. Fernando Alzate (Biology institute, Universidad de Antioquia, Medellin, Colombia). A voucher specimen (no. Alzate-50622) was kept at University of Antioquia herbarium. The *I. paraguariensis* used was a commercial sample from Corrientes, Argentina.

Preparation of extract

The *I. laurina* and *I. paraguariensis* infusion was prepared using 13 g of dry leaves in 500 mL of distilled and boiled water with constant stirring for 30 min, filtered, freeze-dried and stored at -20 °C in plastic tubes, sealed and protected from light until use.

Cell culture

SW480 and SW620 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured in medium DMEM supplemented with 10 % HS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 % non-essential amino acids. For all experiments, cells were switched to assay medium containing 3 % HS, and ITS 1 % for treatments 24 h after seeding [8].

Determination of total flavonoid content (TFC)

Four mL of distilled water was added to 1 mL of each infusion followed by 5 % (w/v) sodium nitrite solution and 10 % (w/v) aluminium chloride solution. After incubating for 5 min at Room Temperature (RT), 1 M NaOH was added. Absorbance was read at 510 nm. Results were expressed as mg gallic acid equivalents (GAE) in 100 g of dry extract, from a gallic acid calibration curve [9].

Determination of total phenolic content

Folin-Ciocalteau reagent (125 µL) and distilled water (625 µL) were added to 1 mL of each infusion, after incubating for 6 min at RT, 70 g/L Na_2CO_3 was added, mixed and incubated for 90 min at RT. The absorbance was read at 760 nm [10]. The results are expressed as described for TFC assay.

Determination of caffeoyl derivatives

Hydroxycinnamic acids were analyzed by high-performance liquid chromatography with photodiode array detection (HPLC–DAD) using a Shimadzu LC-20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II) C18 column (5 µm) 250 × 4.6 mm (Restek ©, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). Chlorogenic (≥ 95 %), caffeic (≥ 98 %), ferulic (≥ 99 %), and p-coumaric acids (≥ 98 %), were adopted as the standards for the identification and quantification of hydroxycinnamic acids at 320 nm. The mobile phase was a mixture of acetonitrile (10 µL), acidified water (phosphoric

acid at pH 2.5) (40:60) v/v, at a flow rate of 0.8 ml/min [11].

Oxygen Radical Absorbance Capacity (ORAC) assay

This method measures the antioxidant scavenging activity of infusions against peroxyl radical generated by thermal decomposition of 2,2 - azo-bis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C [12]. Fluorescein (FL) was used as the fluorescent probe. Reduction in FL fluorescence (excitation: 493 nm; emission: 515 nm) was an indication of the extent of damage from its reaction with the peroxyl radical. Infusions and solutions of 10 mM AAPH, 70 nM fluorescein and Trolox were prepared in a 75 mM phosphate buffer pH 7.4. The antioxidant activity was expressed mmol Trolox/100g dry extract from a Trolox calibration curve. ORAC of infusions was measured by assessing the area under the fluorescence decay curve (AUC) relative to that of a blank. These areas were employed to obtain ORAC values, according to equation 1:

$$ORAC = [(AUC-AUC^{o})/AUC_{trolox}-AUC^{o})] \times f \times [trolox] \ldots\ldots (1)$$

where AUC = area under curve of infusion; AUC^{o} = area under curve for the control; AUC_{trolox} = area under curve for trolox. f = dilution factor, and [trolox] = Trolox molar concentration.

Ferric Reducing Antioxidant Power (FRAP) assay

Based on the increased absorbance due to the formation of TPTZ-Fe (II) complex in presence of reducing agents, aliquots of infusions were mixed with FRAP reagent at RT. The absorbance was measured at 595 nm. Ascorbic acid was used for the calibration curve and results were expressed as mmol ascorbic acid/100 g dry extract [10].

3-(4, 5-Dimethylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide (MTT) assay

Cytotoxic activity of infusions was screened in 3000 viable cells seeded in a 96-well plate and treated for 48 h with the infusions at 0 - 200 µg/mL. Thereafter, 5 mg/mL MTT solution were added to each well, and the wells were incubated in darkness (37 °C, 4 h). The formazan crystals were dissolved by using acidified isopropanol (0.4 N HCl). The amount of MTT-formazan is proportional to the number of living cells and was measured at 540 nm and at 750 nm [13]. The concentration of infusions that caused 50 % of growth inhibition (IC50) was calculated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

Sulforhodamine B (SRB) assay

Cells were cultured as described for MTT assay. Dulbecco's modified Eagle's medium (DMEM) 3% supplemented horse serum was replaced every 48 h with different concentrations of infusions. Cell culture was stopped by addition of trichloroacetic acid (50 % v/v) at 4 °C for 1 h, and cell proteins were stained with 0.4 % (w/v) SRB, absorbance at 490 nm is proportional to the number of adherent and live cells [13].

Cell death analysis

PI was used to detect and measure the percentage of cell population in the SubG0/G1 region corresponding to the amount of dead or dying cells [14]. After treatments cells were harvested by trypsinization, fixed in methanol:PBS (9:1, v/v) at -20 °C for 30 min, washed and re-suspended in PBS containing 0.25 mg/mL RNAse A and 0.1 mg/mL PI, incubated in darkness (37 °C, 30 min). The fluorescence of 10,000 cells was analyzed in EPICS XL flow cytometer (Coulter, Hialeah, Florida), and the Windows Multiple Document Interface 2.8 Software (WinMDI, Scripts Research Institute, La Jolla, CA.).

Detection of apoptosis

Apoptosis was quantified by measuring phosphatidylserine externalization using a flow cytometer [14]. After 48 h of treatment, cells were harvested by trypsinization and annexin-V–FLUOS staining kit was used according to the manufacturer's instructions. The fluorescence of 10,000 cells was analyzed in EPICS XL flow cytometer (Coulter, Hialeah, Florida), and the Windows Multiple Document Interface 2.8 Software (WinMDI, Scripts Research Institute, La Jolla, CA.).

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). The ANOVA test followed by the Bonferroni test's ($p < 0.05$) was used. Data were analyzed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego California, USA).

RESULTS

Polyphenolic compounds and antioxidant activity

As shown in Table 1, *I. laurina* infusion contained higher amount of total phenols and hydroxycinnamic acids than *I. paraguariensis*,

but a similar flavonoid content. In Table 2, the *I. laurina* infusion present better reducing power (value FRAP) and antioxidant capacity measured by proton transfer mechanism hydrogen atom transfer (ORAC value).

Effect of *I. laurina* and *I. paraguariensis* infusions on cell viability, cell growth and cell cycle

As shown in Figure 1, the inhibitory effect on SW480 and SW620 cell viability increased in a dose-dependent manner. The IC_{50} values for *I. laurina* infusion and *I. paraguariensis* on SW480 were 113.2 and 143.1 µg/ml, respectively. The IC_{50} for I. laurina infusion and I. paraguariensis on SW620 cells were 115 and 133.4 µg/ml, respectively.

The effect of both infusions on SW480 and SW620 cell growth is shown in Figure 2. The optical density (OD) of SW480 cell protein decreased between 33.7 and 89.1 % with *I. laurina* infusion at 25 - 200 µg/ml. A comparable effect in SW480 cells was observed with *I. paraguariensis* infusion at 25 - 200 µg/ml where OD decreased between 29.6 and 79.9 %. Similar results were obtained in SW620 cells at the same concentrations of *I. laurina* (32 - 79.2 %) and *I. paraguariensis* (26 – 70 %).

The result observed with each infusion (150 µg/mL) on SW480 and SW620 cell cycle is shown in Figure 3. Both infusions increased the subG0/G1 population after 48 h of treatment. This population was enhanced by 38 % (*I. laurina*) and by 33 % (*I. paraguariensis*) compared to control in SW480; whereas subG0/G1 was enhanced by 16 % (*I. laurina*) and by 19 % (*I. laurina*) in SW620 cells compared to control.

***Ilex laurina* and *Ilex paraguariensis* infusions induced apoptosis in SW480 and SW620 cells**

We questioned whether these infusions inhibited SW480 and SW620 cell growth and induced enhanced of SubG0/G1 population through apoptosis. As shown in Figure 4, both infusions induced apoptosis in SW480 and SW620 cells compared to the respective non-treated cells (control). *I. laurina* induced 20 % of SW480 and 25 % of SW620 early apoptotic cells. A similar result was obtained with *I. paraguariensis* infusion (SW480: 28 %; SW620: 30 % early apoptotic cells).

DISCUSSION

Although many dietary compounds have been identified to be able to interfere with colorectal carcinogenesis by different mechanisms (antioxidant, antiproliferative, pro-apoptotic), this is the first report showing the antioxidant activity of an infusion of dried leaves from *I. laurina* and their antiproliferative and apoptotic effects against two colon cancer cells by reducing cell growth, inducing SubG0/G1 population and apoptosis in a similar way to the YMT.

Phenolic compounds and flavonoids in plants may confer antioxidant activity by acting as free radical scavengers, reducing agents, singlet oxygen quenchers, hydrogen donors, and chelating agents of metal ions [15]. The ORAC values obtained here showed that the antioxidant activity of *I. laurina* by scavenging peroxyl radicals was better than YMT and other *Ilex* species [5].

In a similar way, FRAP values suggest that *I. laurina* may act as an electron donor and may react with free radicals transforming them into

Table 1: Polyphenolic compounds of *Ilex laurina* and *Ilex paraguariensis* infusions

Sample	Total phenolics	Flavonoids	Caffeoyl derivatives (mg/L)			
	mg GAE/100g dry extract	mg GAE/100g dry extract	Chlorogenic acid	Caffeic acid	p-Coumaric acid	Ferulic acid
I. laurina	23.70 ± 0.18	4.55 ± 0.16	429.22 ± 20.23	52.59 ± 18.78	47.32 ± 2.43	21.51 ± 1.64
I. paraguariensis	10.78 ± 0.44	4.36 ± 0.20	98.56 ± 4.61	47.40 ± 1.93	24.44 ± 0.85	ND

Data are expressed as mean ± SEM (n =3, p < 0.05) of triplicate determinations

Table 2: Antioxidant capacity of *Ilex laurina* and *Ilex paraguariensis* infusions

Sample/Assay	ORAC value	FRAP value
	(mmol Trolox/100g dry extract)	(mmol Ascorbic acid/100g dry extract)
I. laurina	58.88 ± 0.97	175.11 ± 3.98
I. paraguariensis	35.99 ± 1.67	89.28 ± 1.66

Data are expressed as mean ± SEM (n =3, p < 0.05) of triplicate determinations

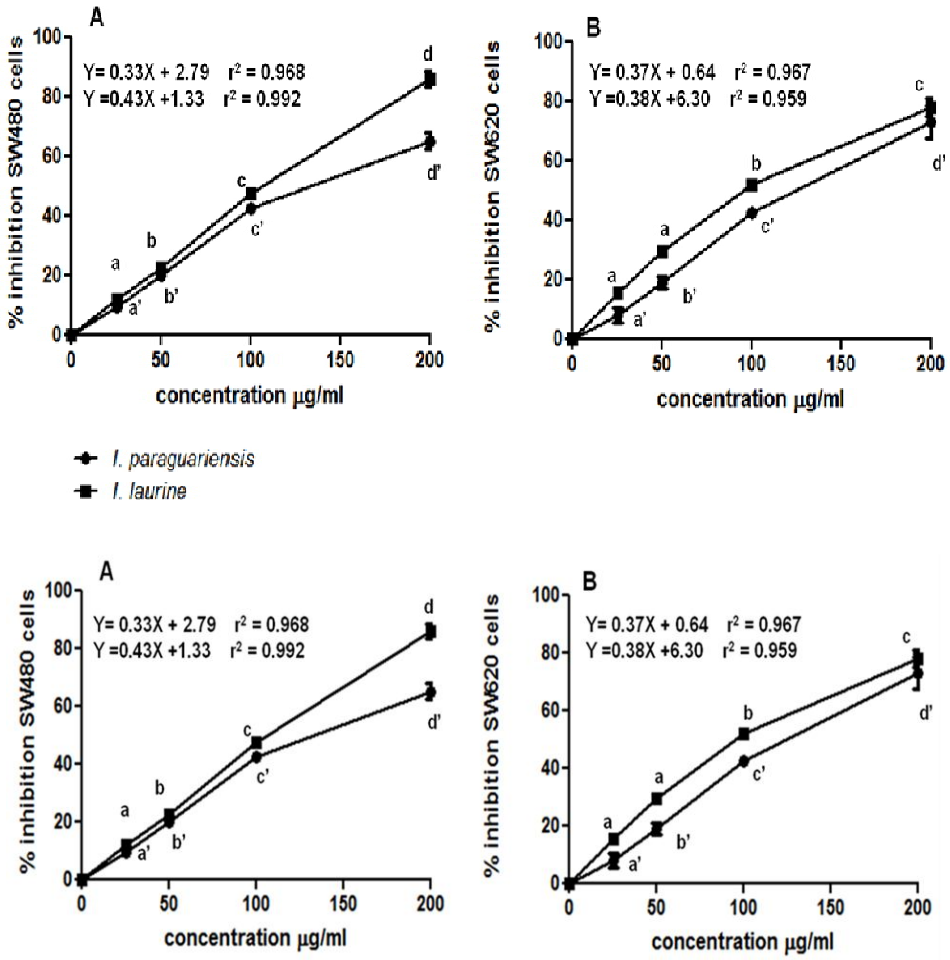


Figure 1: Cytotoxic effects of *Ilex laurina* (▪) and *Ilex paraguariensis* (•) infusions (25 - 200 µg/mL) on SW480 (A) and SW620 (B) cells after 48 of treatment. The values not sharing the same superscript letter differ significantly: a≠b≠c≠d and a'≠b'≠c'≠d'; $p < 0.05$

more stable compounds, comparatively better than YMT. The higher antioxidant activity of *I. laurina* might be explained by the contents of chlorogenic acid which is 4.4-fold higher than YMT. The chlorogenic acid present in YMT is primarily responsible for its antioxidant capacity [16]. It has been reported that chlorogenic acid of 24 samples of commercial YMT confers the highest antiradical activity (91.1 ± 0.04 % at 56 µM) using DPPH method [4], which gives a general idea of the radical quenching ability of the tea samples.

I. laurina infusion exhibited a dose-dependent effect on reducing SW480 and SW620 cell growth. This antiproliferative effect may be associated to cell cycle arrest by increasing hypodiploid cells and apoptotic-early cells. The induction of apoptosis and inhibition of proliferation are widely recognized as chemoprevention mechanisms for CRC, especially apoptosis is considered to be one of the important targets in a preventive approach able to eliminate abnormal cells without affecting living non-malignant cells [2,17].

The components of *I. laurina* infusion responsible of antiproliferative and apoptotic effects are unknown. However, considering that these effects were similar using both plant infusions, they might be attributed to some components such as chlorogenic acid [4] which is 4.4 times higher in the *I. laurina* infusion than YMT. It has been reported that chlorogenic acid induced apoptosis of human oral squamous cell carcinoma (HSC-2), salivary gland tumor cell lines (HSG) [18] and chronic myeloid leukemia cell lines [19] via caspases and mitochondrial dysfunction. Although a little apoptotic effect has been described for this compound on human colon cancer cell lines (HCT15, CO115, COLO 320, SW480 and CaCo-2) [20]. It is known to inhibit the azoxymethane-induced CRC in rats

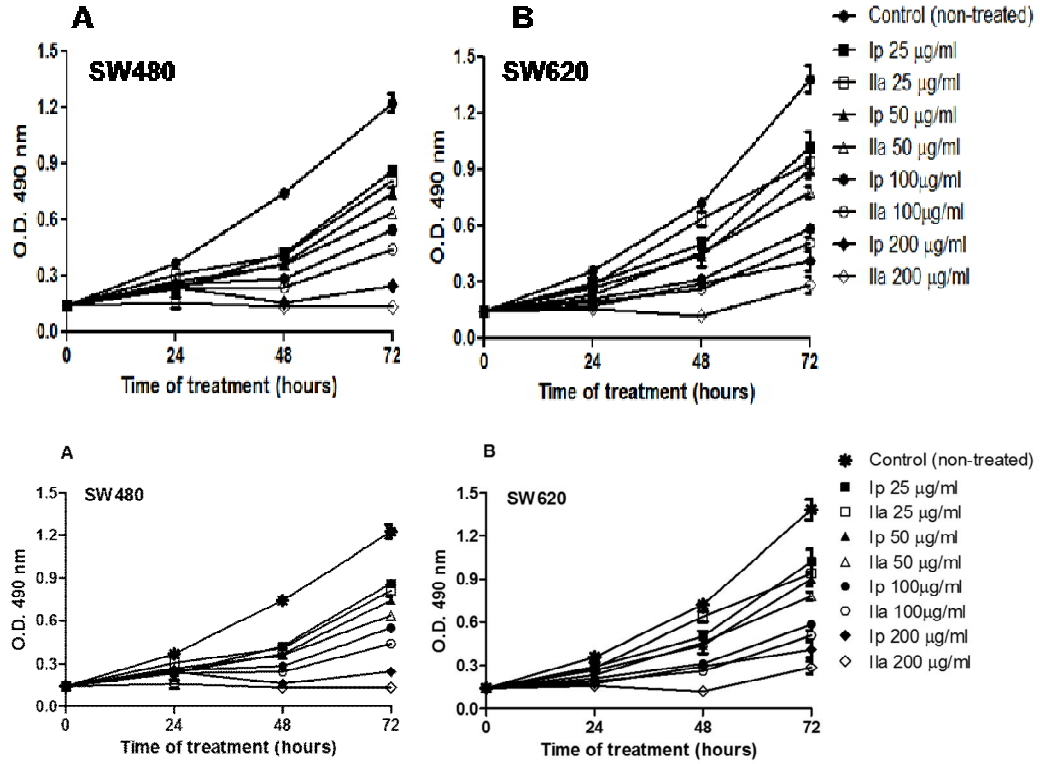
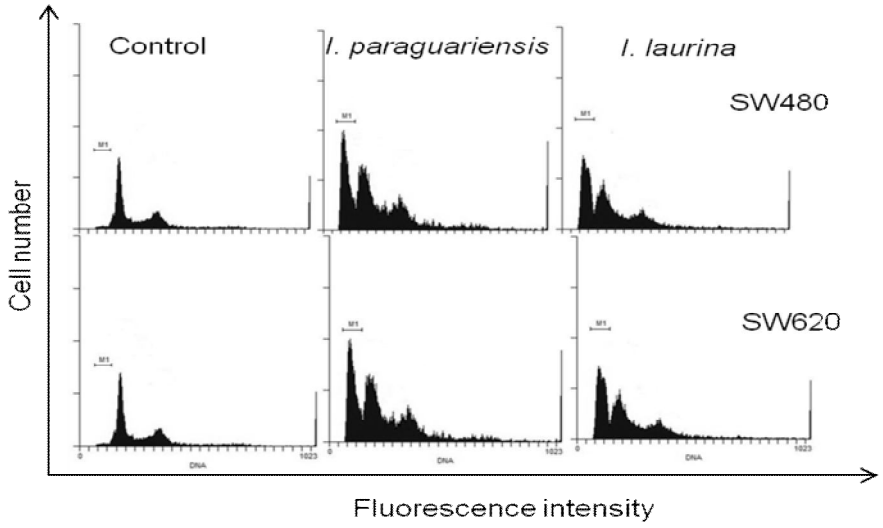


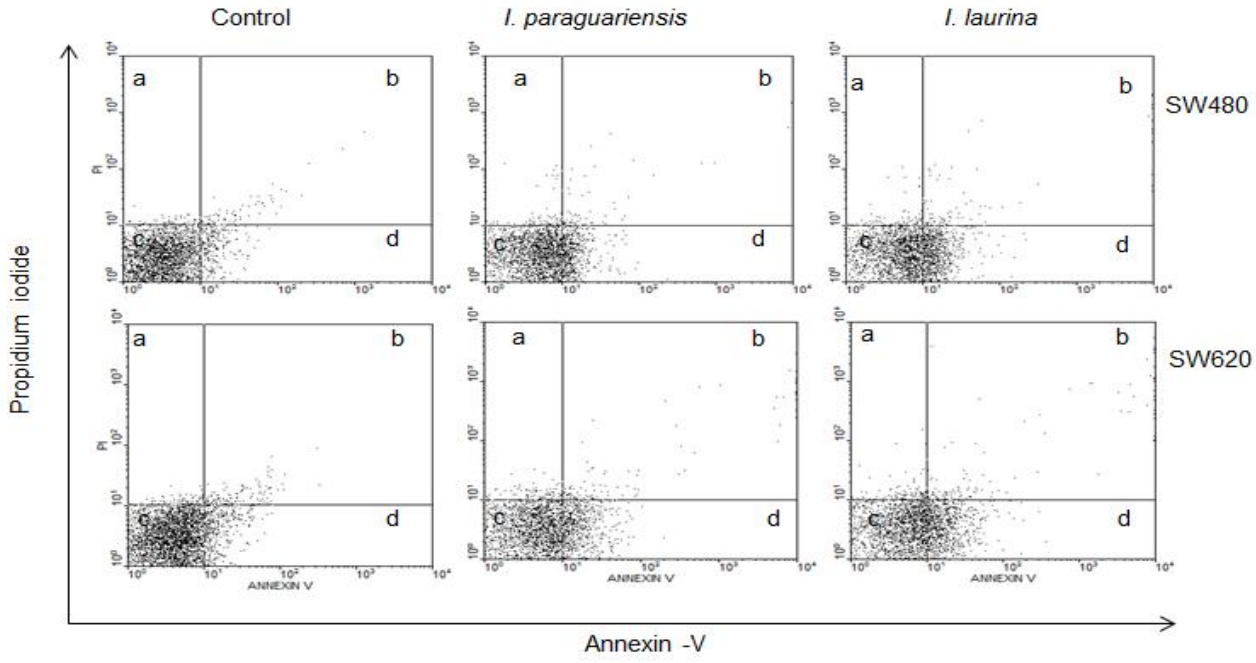
Figure 2: Effect of *Ilex laurina* (IIa) and *Ilex paraguariensis* (Ip) infusions (25-200 µg/mL) on SW480 (A) and SW620 (B) cell growth for 24 h, 48 h and 72 h of treatment. Control: Non-treated cells



% Sub G0/G1 (M1) after 48h

Treatment	SW480	SW620
Control	0.9 ± 0.4	3.7% ± 0.1
Ilex paraguariensis (150 µg/ml)	37.6% ± 1.0*	16.3% ± 0.5*
Ilex laurina (150 µg/ml)	32.8% ± 1.7*	19.2% ± 0.7*

Figure 3: Effects of *I. laurina* and *I. paraguariensis* infusions (150 µg/ml) on SW480 and SW620 cell cycle after 48 h of treatment *$p < 0.05$



	% SW480 cells				% SW620 cells			
	Non-apoptotic	Early apoptotic	Late apoptotic/ necrotic	Dead	Non-apoptotic	Early apoptotic	Late apoptotic/ necrotic	Dead
Control	93.6 ± 0.9	4.9 ± 0.8	0.8 ± 2.0	0.7 ± 2.1	91.0 ± 1.3	6.9 ± 1.9	1.2 ± 2.3	0.9 ± 1.5
Ilex paraguariensis	78.4 ± 3.6*	20 ± 1.9*	0.6 ± 2.8	1.0 ± 0.3	72.6 ± 5.3*	24.8 ± 0.9*	1.2 ± 0.9	1.4 ± 1.8
Ilex laurina	70.3 ± 2.1*	28 ± 1.4*	0.8 ± 2.5	0.9 ± 1.7	67.8 ± 1.7*	29.5 ± 1.6*	1.0 ± 1.8	1.7 ± 2.3

Figure 4: Representative plots of apoptotic effects of *I. laurina* and *I. paraguariensis* infusions (150 µg/mL) on SW480 and SW620 cells after 48 h of treatment; a: dead cells, b: late apoptotic/necrotic cells; c: non-apoptotic cells; d: early apoptotic cells; *$p < 0.05$

[21]. In addition, it is possible that these anticancer effects were due to the synergistic action of chlorogenic acid with other compounds such as ursolic acid and rutin [3,4], also present in YMT. It has been reported that ursolic induced apoptosis on HT-29 cells by suppressing EGFR/MAPK pathway [22], and rutin decreased by 1.2-fold the number of aberrant crypt foci in an azoxymethane-induced CRC in rats [23].

CONCLUSION

I. laurina infusion exhibits antioxidant, antiproliferative and apoptotic effects comparable to a commercial YMT on a human colon adenocarcinoma cell line and their metastatic-derived cell line. The presence of phenolic acids, chlorogenic acid and comparable concentrations of flavonoids to YMT suggest that these properties might be attributed partly to these compounds.

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