

Full Length Research Paper

## Antioxidant and cytotoxic activity of black and green tea from *Vaccinium meridionale* Swartz leaves

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Tea is a beverage made from leaves with high contents of polyphenolic substances that vary based on the process they are subjected to. In this study, the apical and young leaves from *Vaccinium meridionale* (named mortiño) were processed to obtain two kinds of tea: green and black tea. This was done in order to compare their antioxidant activity, content of secondary metabolites at different temperatures of extraction and their antiproliferative effect against SW480 colon cancer cells. Results showed that at 40°C, the green tea infusion presented higher antioxidant activity than the black tea infusion, based on their evaluation using Trolox equivalent antioxidant capacity (TEAC)-diphenylpicrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) techniques. The green tea also had maximum contents of epicatechin, caffeine, ferulic, chlorogenic and ascorbic acid than the black tea. The total contents of phenols, including hydroxycinnamic acids (caffeic and p-coumaric acid) presented similar results, in both types of tea at the same temperature, as well as the total contents of flavonoids and catechin. When temperatures increased, the extraction of bioactive compounds was more efficient in the black tea infusion than the green tea. This situation led to the increased growth rate per the temperature of the total content of phenols, among which chlorogenic, caffeic and p-coumaric acid were prominent, as well as the corresponding non-polyphenolic substances such as ascorbic acid. The latter may be responsible for the increased antioxidant activity as the temperature increased in the extraction. This antioxidant activity was observed in the black tea from mortiño leaves, using TEAC-DPPH, fluorescence recovery after photobleaching (FRAP) and ORAC assays. Both types of teas had a dose-dependent antiproliferative effect against SW480 colon adenocarcinoma cells. The IC<sub>50</sub> of the green and black tea was 26.3 and 36 µg/ml, respectively. These findings suggest that a tea prepared from mortiño leaves may be a promising source of antioxidant and bioactive compounds against colon cancer cells.

**Key words:** Antioxidant, antiproliferative, mortiño, polyphenols.

### INTRODUCTION

*Vaccinium meridionale* Swartz is a native Colombian plant belonging to the family of Ericaceae. The fruit is commonly known as mortiño or agraz; it is a dark purple

globose berry when ripe. This fruit has a high potential for domestic consumption and has been included in the list of species with outward market, called "potential new

berry", "Andean blueberry" or "Colombian blueberry". There is a growing interest in this fruit that has been as considered a functional food due to its content of anthocyanins and other polyphenols. Garzón et al. (2010) evaluated the chemical composition, anthocyanin, non-anthocyanin phenolics and phenolic composition of mortiño. Cyanidin 3-galactoside was the major anthocyanin, while the most abundant non-anthocyanin phenolic was chlorogenic acid.

Gaviria et al. (2009) evaluated the content of phenols, anthocyanins and antioxidant activity by different methods. They found similar or higher values than those reported for other species of *Vaccinium*. Moreover, non-ethanolic extracts of *V. meridionale* Swartz rich in anthocyanins showed cardioprotective activity in rats during an ischemia-reperfusion process mediated by reactive oxygen species (ROS) (Lopera et al., 2013). Maldonado et al. (2014) studied the antioxidant activity of mortiño berry in aqueous extracts using fluorescence methods. They found it has the ability to trap total ROS and reactive nitrogen species (RNS), peroxy, hydroxyl radicals; it has effects on the viability and growth of primary tumor cells of colon cancer (SW480) and their metastatic-derived cells (SW620), which are considered as *in vitro* model representing colon cancer progression to metastatic disease. However, there are no reports on the nutraceutical potential of the leaves, specifically in aqueous tea infusions.

Tea is a beverage known worldwide, prepared from hot water infusion of the leaves of *Camellia sinensis* L. (Cabrera et al., 2006). Tea consumption is similar to water and greater than coffee, beer, wine and beverage (Rietveld and Wiseman, 2003). This product is classified according to the type of process the leaves undergo to get the infusion as: green or unfermented tea, oolong or semi-fermented tea and black or fermented tea.

The production of green tea is characterized by a stage of fresh leaf wilting, followed by a coiled and short heating process at 300°C, designed to inactivate the polyphenol oxidase enzyme and generating native microflora of catalysis and aerobic oxidation. But, the processes of black and oolong tea are characterized by the development of four stages: first, leaf wilting where humidity is decreased and proteins are broken down by the action of the protease enzyme that can generate an increase in amino acid. Also, oxidation of lipids can occur, causing the level of unsaturated fatty acids to decrease by oxidative cleavage to form aromatics, contributing to the development of the aroma, color and flavor (Chen et al., 2011; TomLins and Mashingaidze, 1997). At this stage, the chlorophylls are degraded by 15%. In this regard, our green tea infusions showed a bottle-green colors, fresh flavors and herbaceous tastes.

In addition, the black tea infusions showed a darker green colors than the previous ones, close to dark bole, and intense tastes and flavors.

The second stage is the leaf roll in which the polyphenols in vacuoles are mixed with polyphenol oxidase enzyme in the cytoplasm. This leads to the third step, fermentation, where polyphenoloxidases and other enzymes of the indigenous microflora are transformed in flavanols and polyphenol theaflavins and thearubigins, which also contribute to the dark color of black tea (Kuhnert et al., 2010) and reduce the astringency and flavor taste characteristic of this vegetal species (Wang et al., 2000). This stage is controlled and stopped according to the requirements of loss of green color (Cabrera et al., 2006). The last stage that is presented is drying, which helps to transform chlorophyll to pheophytin (Ramasamy et al., 2013).

Tea is recognized as an essential source of bioactive compounds such as (+) catechin, (-) epicatechin, (-) epigallocatechin and (-) epicatechin gallate, among others. They contribute to the antioxidant activity of this drink and the organoleptic properties (Fernández et al., 2000; Kim et al., 2011). Also, this type of drink is known for its action against diseases such as cancer, in addition to its pharmaceutical activity due to its antihypertensive, anti-atherosclerotic and hypolipidemic properties.

These properties may be attributed to antioxidant activity from polyphenols such as flavonoids (Chen et al., 2001). In this regard, a freshly prepared infusion of green tea contains 30 to 42% of catechin in weight; in black tea, the aqueous extract of the dried material contains between 3 and 10% of catechin, 2 and 6% of theaflavin and over 20% of thearubigin (Lambert and Yang, 2003). Additionally, natural sources contain other compounds such as tannins, vitamins and terpenoids which have similar characteristics with those in the phenol compounds (Exarchou et al., 2002) biological properties. It is necessary to note that the variety, weight, presentation and technical processing affect the estimation of these flavonoids.

The mode of action of these bioactive substances is based on redox mechanism, which these compounds exert as reducing agents; therefore, they can scavenge or quench reactive oxygen and nitrogen species, including free radicals such as superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl (OH) and nitric oxide (NO), as well as other species such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and nitrous acid (HNO<sub>2</sub>) (Oh et al., 2013; Zhu et al., 2002). Tea polyphenols have antiproliferative activity and induce apoptosis against cancer cell lines and animal studies (Yang et al., 2011; Cordero-Herrera et al., 2013). Based on these considerations, the aim of the research was to determine and compare the content of bioactive

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metabolites, antioxidant activity and antiproliferative effect of two infusions of leaves from mortiño prepared as green and black tea extracted at different temperatures. Taking together the results obtained, let us suggest the nutraceutical potential of these beverages.

## MATERIALS AND METHODS

### Chemicals and equipment

Diphenyl - 1 - 2,2 picrylhydrazyl (DPPH), 6 - hydroxy - 2,5,7,8 - tetramethyl chromane - 2 - carboxylic acid (trolox), 2,4,6 - tris (2 - pyridyl - s - triazine) (TPTZ), 3,4,5 - trihydroxybenzoic acid (gallic acid), folin ciocalteu, sodium carbonate, L-ascorbic acid, methanol, formic acid, iron chloride, 3.6 buffer, pH 1 buffer, pH 4.5 buffer, pH 7.4 buffer, fluorescein and 2,2'-azobis (2-amidino-propane) (AAPH) were obtained from Merck (Germany). The water used in the experiments was bi-distilled type. Vis Jenway 6405, a spectrophotometer brand Perkin Elmer LS 55 precisely and Shimadzu brand high-resolution chromatography high performance liquid chromatography (HPLC)-ultra violet (UV) spectrophotometer were used.

### Plant sample collection

Leaves were harvested at the Catholic University in Rionegro, Antioquia, Colombia. The leaves were collected manually and selected on the basis of youthfulness and position in the tree. Younger leaves near the apical bud were selected. This material has a voucher number ILS 14050070. Samples were transported to laboratory in plastic bags sealed and washed with distilled water.

### Green tea manufacturing

The elaboration process for obtaining both green tea and black tea from the leaves of *V. meridionale* S. was the same as cited by Gil (2010) from leaves of *C. sinensis* L., so there is no definite via for obtaining.

In the case of green tea, the process carried out on the leaves of *V. meridionale* S. consisted of several steps: first, leaves were subjected to a drying process in an oven at 48°C, for 1 h, in order to remove about 30% of their original moisture, which was previously determined on an average value of 62.7%; the obtained leaves were manually rolled and dried in an oven at a temperature between 90°C for 1 h, with the initial goal of reducing the moisture content that remained after step 1 to 4%. However, it resulted to only 13.2%. Subsequently, the plant material obtained was crushed and put into tea bags, after which the infusion was performed by dipping them in 50 ml of water at 40, 60, 80, 90 and 100°C for 10 min. Then, we proceeded to evaluate the antioxidant activity of the tea obtained via the Trolox equivalent antioxidant capacity (TEAC) techniques-DPPH, fluorescence recovery after photobleaching (FRAP) and oxygen radical absorbance capacity (ORAC), to determine the ascorbic acid, phenols and total flavonoids content, and to identify and quantify the amount of catechin, epicatechin and caffeine, hydroxycinnamic acids; they were chlorogenic, caffeic, ferulic and p-coumaric acid and sugars, among which glucose, fructose and sucrose were considered.

### Black tea manufacturing

In the case of black tea, the process carried out on the *V. meridionale* S. leaves consisted of several stages: first, leaves were

subjected to a drying process in an oven at 48°C, for 1 h, in order to remove about 30% of their original moisture which was previously determined (57%). Subsequently, the leaves obtained were manually rolled, and subjected to a process of "fermentation", in which the leaves were placed in contact inside a sealed chamber, with oxygen in an atmosphere characterized by 95% moisture, generated by a potassium nitrate solution at 27°C. The leaves were weighed at 2 and 5 h after the start of the "fermentation" process. They were hydrated until approximately 54% humidity. With these features, the leaves were subjected to a drying oven at 90°C for a certain time, for two purposes: firstly, to inactivate the activity of the polyphenol oxidase enzyme and secondly to reduce the moisture. The moisture content was approximately 1%. Subsequently, the plant material obtained was crushed and put into tea bags, after which the infusion was performed by dipping them in 50 ml of water at 40, 60, 80, 90 and 100°C for 10 min. Then proceeded to evaluate the antioxidant activity of the tea obtained via the TEAC techniques-DPPH, FRAP and ORAC, to determine the content of ascorbic acid, phenols and total flavonoids, and to identify and quantify the content of catechin, epicatechin and caffeine, hydroxycinnamic acids; they were chlorogenic acid, caffeic, ferulic and p-coumaric acid, and sugars, among which glucose, fructose and sucrose were considered.

### DPPH method

Radical scavenging activity against the stable radical DPPH was measured using the methods of Brand-Williams et al. (1995), with certain modifications. The method is based on the reaction of 10 ml of sample with 990 ml of DPPH solution for 30 min at room temperature, followed by determining the decrease in absorbance at 517 nm associated with a reduction in the DPPH concentration. The results were expressed in units, TEAC.

### FRAP assay

The antioxidant capacity of tea was estimated according to the procedure described by Benzie and Strain (1996), with some modifications. This method is based on the increase in absorbance due to the formation of 2,4,6-tripyridil-s-triazine (TPTZ)-Fe (II) in the presence of reducing agents. The FRAP reagent contained 2.5 ml of 10 µM TPTZ in 40 mM HCl. FeCl<sub>3</sub> (2.5 ml of 20 µM) and acetate buffer (25 ml of 0.3 µM, pH 3.6) were freshly prepared and warmed to 37°C. A volume of 50 µl of extract was mixed with 950 µl FRAP reagent already dissolved in acetate buffer (pH 3.6). The absorbance increase was measured at 590 nm. The FRAP values were expressed as AEAC (ascorbic acid equivalent antioxidant capacity: mg ascorbic acid per g dry powder) using an ascorbic acid standard curve.

### ORAC assay

The ORAC assay was determined using the following methodology: 3 ml was prepared from the following solution: 21 µl of a 10 µM solution of fluorescein, 2899 µl of 75 mM phosphate buffer (pH 7.4), 50 µl of 600 mM 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 30 µl of extract. Fluorescence was recorded on a Perkin Elmer LS45 spectrophotometer with a thermostated multicell. The results were expressed as micromoles of Trolox® equivalents per 100 g lyophilized according to Equation 1.

$$ORAC = \frac{(AUC - AUC^0)}{(AUC_{Trolox} - AUC^0)} f[Trolox] \quad (1)$$

where AUC is the area under the curve of the sample,  $AUC^{\circ}$  is the area under the curve for the control,  $AUC_{Trox}$  is the area under the curve for Trolox® and  $f$  is the dilution factor extracts (Romero et al., 2010).

### Total phenols

The total phenolic content was determined according to the adapted Folin–Ciocalteu method (Singleton and Rossi, 1965). The extracts (50 µl) were mixed with 125 µl of Folin–Ciocalteu reagent and 400 µl of sodium carbonate solution (7.1% p/v), and the resulting solution was brought to a final volume of 1000 µl. The mixture was stirred and stored at room temperature for 30 min in the dark. The absorbance was measured at 760 nm against a blank. Aqueous solutions of gallic acid were used for calibration. The results are expressed as gallic acid equivalents (GAE)/g dry powder.

### Total flavonoids

The flavonoids were determined by colorimetric method (Marinova, 2005). The extracts (100 µl) were mixed with 30 µl of  $NaNO_2$  (5% p/v), 30 µl of  $AlCl_3$  (10% p/v), 200 µl of NaOH (1M) and the resulting solution was brought to a final volume of 1000 µl with distilled water. The absorbance was measured at 510 nm. The results are expressed as catechin equivalents/g dry powder.

### Hydroxycinnamic acids determination by HPLC- diode array detector (DAD)

Hydroxycinnamic acids were analyzed by direct injection of the samples, previously filtered through a 0.45 µm pore-size nylon filter, in a HPLC-DAD using a Shimadzu LC- 20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II)  $C_{18}$  column (5 µm) 250 × 4.6 mm (Restek®, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). Chlorogenic, caffeic, ferulic and p-coumaric acids were adopted as the standard for identification and quantification of hydroxycinnamic acids at 320 nm. The mobile phase was a sample of 10 RL of a mixture of acetonitrile, acidified water (phosphoric acid at pH = 2.5) (40:60) v/v, supplied at a rate of 0.8 ml/min (Kelebek et al., 2009).

### Catechin and epicatechin determination by HPLC-DAD

(+) - Catechin and (-) - epicatechin were analyzed by direct injection of the samples, previously filtered through a 0.45 µm pore-size nylon filter, in a HPLC-DAD using a Shimadzu LC- 20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II)  $C_{18}$  column (5 µm) 250 × 4.6 mm (Restek®, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). (+) - Catechin and (-) - epicatechin were adopted as the standard for identification and quantification at 280 nm. The mobile phase was methanol (A) acidified water (0.1% formic acid) (B) with gradient elution of 0.01 min 60% A was used; 5 to 12 min 80% A; 13 to 14 min 60% A. Flow rate of mobile phase was 1.0 ml/min (Oliveiro et al., 2009).

### Caffeine determination by HPLC-DAD

Caffeine was analyzed by direct injection of the samples, previously filtered through a 0.45 µm pore-size nylon filter, in a HPLC-DAD using a Shimadzu LC- 20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II)  $C_{18}$  column (5 µm) 250 ×

4.6 mm (Restek®, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). Caffeine was adopted as the standard for identification and quantification at 280 nm. The mobile phase was methanol. It was used in isocratic mode working at a flow of 1.0 ml/min (Brunetto et al., 2007).

### Cell culture

SW480 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured according to a previously described procedure (Maldonado et al., 2014). Cells were cultured in 75 cm<sup>2</sup> Falcon flasks with Dulbecco's modified eagle's medium supplemented with 25 mM glucose, 2 mM L-glutamine, 10% heat (56°C)-inactivated horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% non-essential amino acids. Incubations were carried out at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The culture medium was replaced every 48 h. For all experiments, horse serum was reduced to 3%, and the medium was supplemented with 10 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium (ITS defined medium). Cells were exposed to different extracts for 24 h after seeding.

### Sulforhodamina B (SRB) assay

The effect of extracts on growth cells was studied by using the SRB assay according to Gossé et al. (2005), a colorimetric assay based on staining of total cellular protein from cells with SRB dye. In brief, 3000 viable cells from each cell line were exposed to extracts for 24 h after seeding and incubated for different times. Control cells were treated with 0.1% dimethyl sulphoxide (DMSO). Culture media were replaced every 48 h. The cell culture was stopped by the addition of trichloroacetic acid (50% v/v), and cell proteins were determined by staining with 0.4% (w/v) SRB (Sigma-Aldrich, United States). The relationship between cell number (protein content/well) and absorbance is linear from 0 to  $2 \times 10^5$  cells per well. All experiments were performed in triplicate. The concentration able to kill 50% of cells ( $IC_{50}$ ) was calculated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The absorbance of control group (non-treated cells) was considered as 100% viability. The percent inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (OD_t / OD_c)] \times 100$$

Where  $OD_t$  is the optical density (OD) of treated cells, and  $OD_c$  for control (non-treated cells).

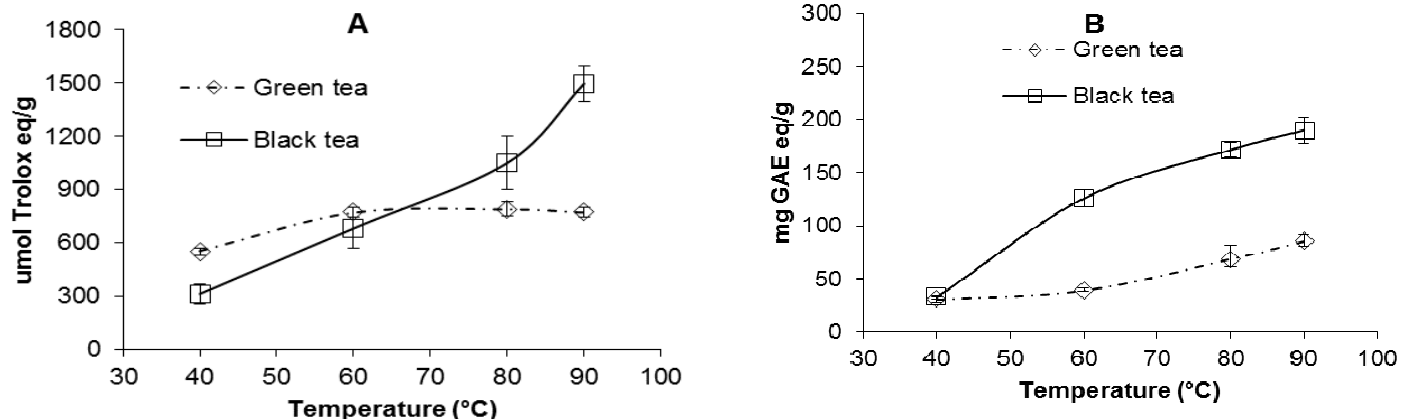
### Statistical analysis

The variables were characterized in terms of the extraction temperature, using the Statgraphics Centurion XVI statistical program. Analysis of variance (ANOVA) was applied to each variable depending on the stages of development, with a significance level of 5%.

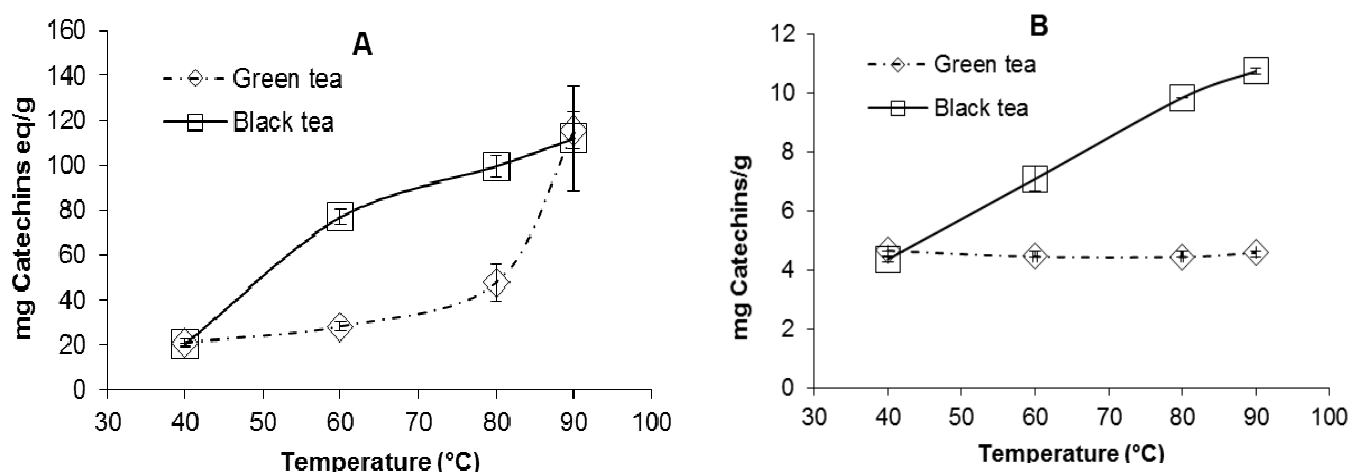
## RESULTS

### Antioxidant activity and secondary metabolites

Figure 1A and B show the effect of temperature on the peroxy radical scavenging capacity (ORAC) and the content of total phenols, respectively, both aqueous extracts of green tea and black tea. In green tea, the



**Figure 1.** Effect of temperature on ORAC values (A) and total phenol content (B) in green and black tea.



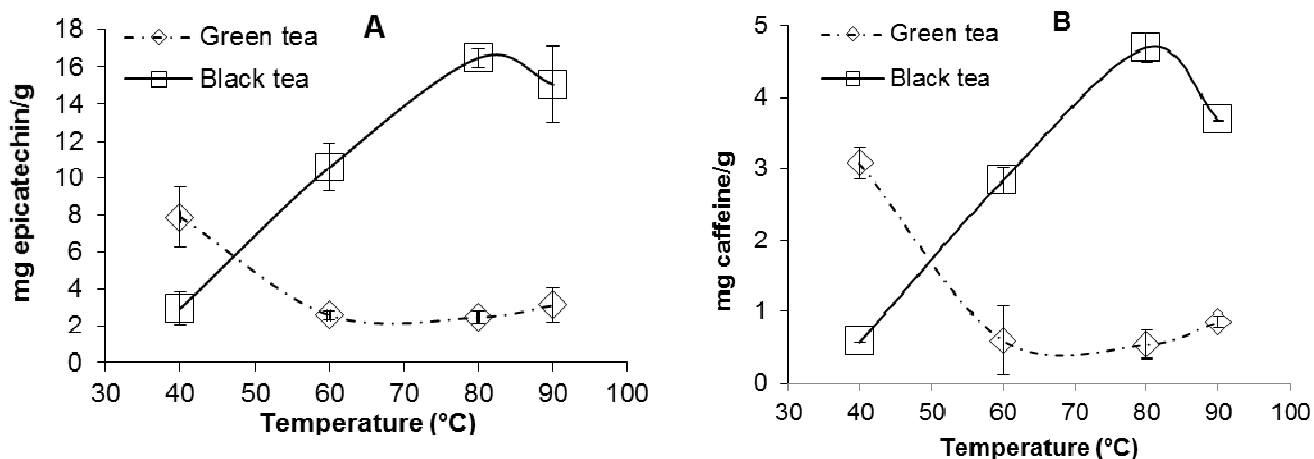
**Figure 2.** Effect of temperature on total flavonoids (A) and catechin content quantified by HPLC (B).

results obtained from the first technique varied significantly between 40 and 60°C, with values of  $542.04 \pm 18.02$  and  $778.8 \pm 27.22$   $\mu\text{mol Trolox/g}$  of dry leaf, respectively. Subsequently, the values remained nearly constant in the temperature range between 60 and 90°C. However, the values respective to black tea aqueous extracts increased significantly throughout the whole period of temperature, obtaining values of  $313.65 \pm 37.06$ ,  $682.16 \pm 40.34$ ,  $1050.63 \pm 100.45$  and  $1496.82 \pm 101.7$   $\mu\text{mol Trolox/g}$  of dry leaf, for temperatures 40, 60, 80 and 90°C, respectively. The speed rates found for both green and black tea aqueous extracts were 4.3 and 22.4  $\mu\text{mol Trolox}/^\circ\text{C}$  (Table 1), respectively.

According to Figure 1B, the total phenols content in green tea aqueous extracts increased significantly between 60 and 90°C, with values ranging between  $39.28 \pm 1.45$  and  $85.62 \pm 4.9$  mg GAE/g of dry leaf. By contrast, black tea aqueous extracts increased their total phenols content significantly over the extraction

temperatures period, yielding values between 32.66 and  $189.83 \pm 11.9$  mg GAE/g dry leaf, for temperatures of 40 and 90°C. Moreover, the speed rate was obviously higher in black tea aqueous extracts, compared with the green tea, obtaining values of 16.8 and 90.9 mg GAE/ $^\circ\text{C}$ , respectively.

Meanwhile, Figure 2A and B show the extraction temperature effect on the total flavonoids content, expressed as mg catechin equivalents per gram of dried leaf, and mg of catechin per gram of dried leaf but quantified by HPLC technique. In both cases it was observed that content of catechin, corresponding to black tea aqueous extracts increased significantly during the extraction period, with values ranging from  $20.23 \pm 0.70$  to  $112.20 \pm 23.23$  mg catechin equivalents/g dried leaf and from  $4.37 \pm 0.10$  to  $9.72 \pm 0.10$  mg of catechin/g of dry leaf (HPLC). In addition, as shown in Table 1, the speed rate of catechin content in the black tea aqueous extracts was significantly greater than for green tea.



**Figure 3.** Effect of temperature on the content of epicatechin (A) and caffeine (B) in green and black tea. Data are the mean

Figures 3A and B show the extraction temperature effect on the caffeine and epicatechin concentration in aqueous extracts for both kinds of tea. The green tea epicatechin values decreased for extraction temperatures of 40 and 60°C (10.14 and 4.7 mg/g of dry leaf, respectively) and remain constant afterwards. Conversely, black tea epicatechin values increased significantly for each extraction temperatures, with values between 2.96 ± 0.88 and 5.15 mg of epicatechin/g of dry leaf from 40 to 80°C. Both green and black tea aqueous extracts showed opposite behavior respect to the caffeine content; in this case, the black tea caffeine values increased significantly for each extraction temperatures, with values between 0.57 ± 0.02 and 3.68 ± 0.08 mg caffeine/g of dry leaf from 40 to 80°C. By contrast, the caffeine content of green tea aqueous extracts decreased significantly for extraction temperatures 40 and 60°C, with values of 3.08 ± 0.00 and 0.85 ± 0.01 mg of caffeine/g of dry leaf, and remain constant afterwards.

Finally, chlorogenic, caffeic, p-coumaric and ferulic acids were determined by HPLC technique. The green tea aqueous extract values obtained at 90°C were 2.9 ± 0.13, 3.5 ± 0.08, 6.2 ± 1.2 and 3.03 ± 0.02 mg/g of dry leaf, respectively; the black tea aqueous extract values of at 90°C were 3.4 ± 0.04, 4.2 ± 0.05, 7.75 ± 1.1 and 3.7 ± 0.02 mg/g of dry leaf, respectively. Likewise, the antioxidant activities of green and black tea at 90°C were determined by FRAP and DPPH assays obtaining values of 78.8 ± 6.5 and 157.8 ± 18.2 mg ascorbic acid equivalent, respectively, and 491.58 ± 46.56 and 1118.15 ± 171.97 µmoles Trolox/g dry leaf, respectively.

#### Effect of mortiño black tea and green tea on SW480 cell growth

The effect of mortiño black and mortiño green tea on the SW480 cell growth is as shown in Figure 4 where optical density at 490 nm (OD) corresponds to the proteins of the

cells untreated or after treatment with mortiño green or black tea. As shown in Figure 4A, after treatment with mortiño black tea for 72 h, 76.3% of SW480 cell growth (20 µg/ml) was reduced by 58.2% compared to non-treated cells (DMSO 0.1%). While, the effect of green tea on SW480 cell growth at the same conditions was 82.2% at 20 µg/ml and reduced by 49.6% at 200 µg/ml (Figure 4B) compared to non-treated cells.

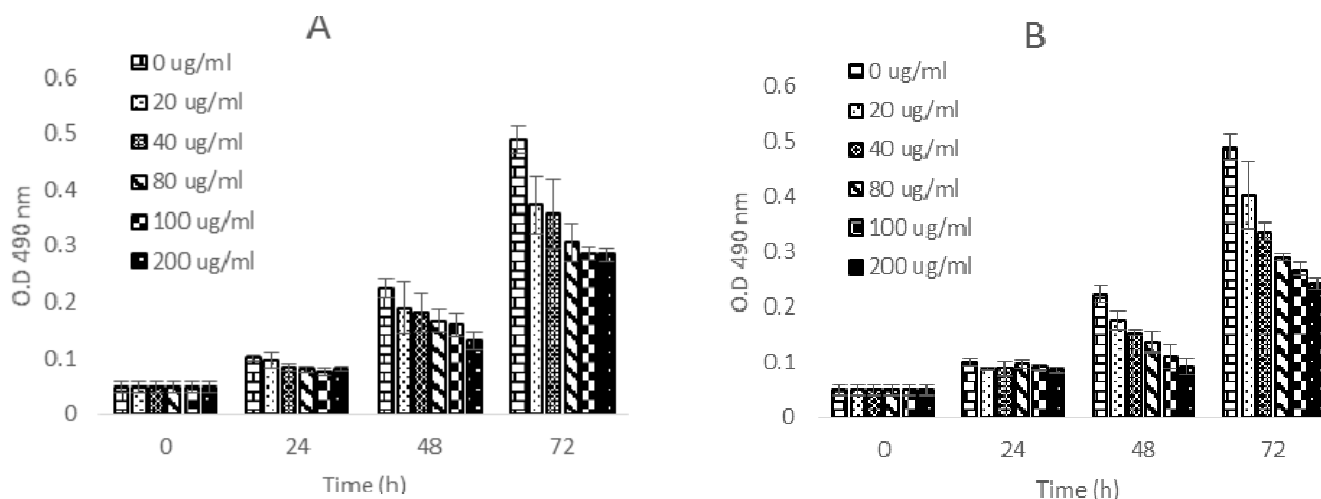
The black tea reduced SW480 cell from 23.7% (20 µg/ml) to 41.9% (200 µg/ml) after 72 h of treatment, while green tea reduced cell viability between 19.5 and 50.4% under the same conditions.

## DISCUSSION

Green and black tea from *V. meridionale* S. leaves contain a large variety of polyphenolic compounds such as catequin, epicatequin and chlorogenic, caffeic, ferulic and p-coumaric acids, and alcaloids such as caffeine. All these compounds determine the antioxidant activity of different aqueous infusions obtained from leaves of this species. To evaluate the antioxidant potential and stability of some metabolites in the processing of green and black tea, monitoring was carried out at different temperatures. Multivariate analysis was made to correlate the extraction temperature effect with the secondary metabolites content in both types of tea. Majority of the test showed statistically significant differences.

Initially, in ORAC technic, green tea showed higher activity than black tea; however, with increasing extraction temperature black tea also increased peroxy radical scavenging capacity. This increased at a rate of 22.4 Trolox µmol/°C, whereas the green tea made a speed of 4.3 µmol Trolox/°C.

Antioxidant activity can be explained by the content of phenolic compounds. The total phenolic contents are similar to the ORAC assay, which shows that phenolic



**Figure 4.** Effect of mortiño black tea (A) and mortiño green tea (B) on the growth of SW480 cells. The cells (3000 cells/well in 96-well plate) were exposed to concentrations of 0 to 200 µg/ml for 24, 48 and 72 h. Control cells were treated with the extract vehicle (dimethylsulfoxide, 0.1% DMSO in culture medium). Data are mean  $\pm$  standard error of the mean ( $n = 3$ ).

**Table 1.** Rate of increase and decrease per temperature.

Technique	Black tea	Green tea
ORAC (umol Trolox)	22.4	4.3
Phenols (mg GAE)	90.9	16.8
Flavonoids (mg catechins)	29.9	30.3
Catechin (mg catechins)	0.1	0.002
Epicatechin (mg epicatechin)	0.3	-0.09
Caffeine (mg caffeine)	0.07	-0.04

compounds from black tea were better extracted than green tea at 90°C. Oh et al. (2013) reported values of  $82.2 \pm 1.8$  and  $82.9 \pm 3.2$  mg GAE/g for green and black tea, respectively at 80°C. These tea values are comparable with matte aqueous infusions that contain  $136.8 \pm 24.8$  mg GAE/g (Mejía et al., 2010; Oh et al., 2013). The values of black tea have a higher growth rate than the corresponding green tea rate.

As expected, the higher the water temperature, the higher the phenolic compounds in tea solution. This is due to the effect of temperature on the solubility and diffusion rate of compounds in the bulk solution. Generally, the solubility and the diffusion coefficient of a substance increase with an increase in the temperature of the solvent (Atkins, 2001). The dissolution and diffusion of caffeine in the aqueous solution, therefore, increased with an increase in water temperature.

At 40°C the two types of tea have similar content of flavonoids; but with gradual increase in extraction temperature; these values grow at different rates. The values obtained are better than those reported in the literature, which shows values of  $16.4 \pm 0.2$  units in green tea and  $14.9 \pm 0.6$  mg catechin equivalents/g of dry leaf

at 80°C (Oh et al., 2013).

Catechin was measured by HPLC and showed at 40°C, similar values of  $4.7 \pm 0.05$  and  $4.4 \pm 0.10$  mg catechin/g dry leaf, respectively for green and black tea. However, at 90°C, black tea was significantly greater than green tea. This behavior is similar to those reported by Carloni et al. (2013), who obtained  $0.3 \pm 0.06$  values for green tea units and  $0.2 \pm 0.03$  units for black tea. The values found for black tea may be due to the enzymatic oxidation of catechin during the process of developing the product generating phenolic pigments such as theaflavins; therefore, there is little effect of the temperature on the increase of this metabolite (Kerio et al., 2013).

For the highest content of epicatechin found in green tea, values decrease with increasing extraction temperature; however, in the black tea epicatechin increased. A similar behavior was observed with the caffeine measured by HPLC, where green tea showed at 40°C a higher value than black tea, decreasing afterwards with the extraction temperature. These results are comparable with the data reported by Carloni et al. (2013), who obtained  $0.6 \pm 0.04$  results for green tea units and  $0.4 \pm 0.05$  units for black tea. In general, the



content of phenolic compounds is dependent on the temperature and time of extraction.

Cytotoxic effect indicated that the green tea was better than black tea on this colon adenocarcinoma cell line. This is consistent with the  $IC_{50}$  value in which for mortiño black tea it was 36.0  $\mu\text{g/ml}$  and for the mortiño green tea it was 26.3  $\mu\text{g/ml}$ . These results indicate that both mortiño black and green tea have a better cytotoxic and antiproliferative effect than other infusions made from green tea (*C. sinensis*) against colon adenocarcinoma cells HT29 ( $IC_{50} = 90.8 \pm 8.3 \mu\text{g/ml}$ ), from yerba mate tea ( $IC_{50} = 17.7 \pm 8.3 \mu\text{g/ml}$  (Mejía et al., 2010) and white tea (*C. sinensis*;  $IC_{50} = 86.68 \pm 0.73 \mu\text{g/ml}$ ) (Hajiaghaalipour et al., 2015). In addition, results obtained here were better than that observed against colon adenocarcinoma CaCo<sup>-2</sup> cells treated with green tea ( $IC_{50} = 161.0 \pm 17.4 \mu\text{g/ml}$ ) and yerba mate tea ( $IC_{50} = 220.0 \pm 12.6 \mu\text{g/ml}$ ) (Mejía et al., 2010). Other authors showed green tea extract (*C. sinensis*) inhibit the growth of two renal carcinoma cell lines with  $IC_{50}$  values of  $54 \pm 10$  and  $129 \pm 28 \mu\text{g/ml}$  for A-498 and 769-P cells, respectively (Carvalho et al., 2010).

According to the criteria of the National Cancer Institute of the United States, an extract is considered active if the  $IC_{50}$  is less than 30  $\mu\text{g/ml}$  on cancer cells (Suffness and Pezzutto, 1990). In this study, the antiproliferative activity of both mortiño black and green tea against SW480 cells was considered to have medium ( $IC_{50} = 36.0 \mu\text{g/ml}$ ) and high ( $IC_{50} = 26.3 \mu\text{g/ml}$ ) cytotoxic and antiproliferative activity, respectively. These results are similar to the evidence obtained with green tea that possess greater efficacy against colon cancer cell lines than black tea. This is attributed to Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea (Li et al., 2013).

Although structure-activity relationship of the compounds analyzed on mortiño green and black infusions was not explored on SW480 cells, Du et al. (2012) reported that flavonoids, such as catechin and epicatechin, did not show antiproliferative effect on SW480 cells. The gallic acid, a phenolic acid, showed some antiproliferative effect. On the other hand, galloylated catechins (catechin gallate, epigallocatechin, epicatechin gallate and EGCG) increased significantly the inhibition of SW480 cell growth compared to catechin and epicatechin. This fact suggests that the two close parallel aromatic rings in galloylated catechins and a third aromatic ring vertical to the two parallel rings may play a key role in their biological activities. However, in this study, Mortiño green and black tea infusions presented a similar content of flavonoids, catechin and hydroxycinnamic acids like caffeic and p-coumaric acid, but green tea infusion contained more epicatechin, caffeine, ferulic, chlorogenic acid than black tea infusion. Thus, the higher cytotoxic and antiproliferative activity of green tea related to black tea may be the result of the synergistic action of these and other unknown compounds present in green tea.

Finally, many mechanisms have been proposed for the inhibition of colon cancer cell growth by tea compounds like the ones obtained here in mortiño green and black tea. The mechanisms proposed include inhibition of MAP-kinases and PI3K/AKT pathways, NF- $\kappa$ B and AP-1 mediated transcription, growth factor-mediated signaling, aberrant arachidonic acid metabolism and proteinase activities (Yang et al., 2011).

## Conclusion

The results suggest that green tea and black tea made from Mortiño leaves can be used as regular drink and they are a rich source of polyphenols, whose concentrations varied depending on the process and extraction temperature. They exhibited greater free radical scavenging capacity at higher temperatures. Also, both types of tea showed cytotoxic and antiproliferative activity with the growth of SW480 cells from colon carcinoma, so that the continuation of this study for evaluation of safety *in vivo* is suggested. The fact that both types of tea inhibit the growth of colon cancer cells *in vitro* makes these kinds of infusions yet unknown potential source of chemopreventive agents that could be taken into account in future research.

## Conflict of Interest

Authors have not declared any conflict of interest.

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