Journal of Medicinal Plant Research

Volume 10 Number 27, 17 July, 2016

ISSN 1996-0875

Academic Journals

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Journal of Medicinal Plants Research

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Vol. 10(27), pp. 402-408, 17 July, 2016
DOI: 10.5897/JMPR2016.6100
Article Number: 26D5A2059544
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Cytotoxic effect and antioxidant activity of Andean berry (*Vaccinium meridionale Sw*) wine

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Received 16 March, 2016; Accepted 30 June, 2016

***Vaccinium meridonale* Sw or Andean berry has antioxidant properties due to its high content of polyphenols, as anthocyanins and phenolic acids. Polyphenols have been associated with the prevention of chronic and cardiovascular diseases. In the last years, alcoholic drinks have been studied for their composition and health benefits. By this, the aim of this research was to obtain three types of alcoholic beverages from Andean berry, which have different treatments. The methods used to obtain the beverages were macerated fruit machine (MAC), preheating of the fruit (CAL) and by combining both of them (MIX). The antioxidant activity was evaluated by FRAP, DPPH, ORAC methods and anthocyanins and total phenols were measured. Finally, the antiproliferative effect was evaluated on a colon cancer cell line (SW480). Findings suggest that ethanol content of final products is not altered by treatment of unfermented Andean berry juice (must). The alcohol concentrations for MAC, CAL and MIX drinks were 90±1.7, 89±3.6 and 94±4.1 g/L, respectively. The results showed that CAL and MIX methods favor the extraction of secondary metabolites and consequently increase the antioxidant activity. The fermentation process affected the antioxidant power and total phenolic content in beverages CAL and MIX. However, no significant changes in these parameters were observed in the MAC drink. These beverages can eventually reduce the cancer cell viability between 15.1 (20 µg/L) and 37.2% (200 µg/mL). Thus, it was concluded that MIX treatment has higher antioxidant power and it could reduce the cancer cell viability.**

Key words: Fermented beverage, Andean berry, antioxidant activity, antiproliferative activity, alcoholic beverage.

INTRODUCTION

The *Vaccinium meridonale Sw* known commonly as “mortiño” or Andean berry belongs to the Ericaceae family with small berries from red to purple color, which are valuable for its antioxidant activity and high

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polyphenols content. Andean berry have been reported to have 329.0 ± 28.0 mg eq cyanidin 3 glucoside/ 100 g FW of anthocyanin, 758.6 ± 62.3 mg eq galic acid/ 100 g FW of total phenols, which show antioxidant power; DPPH (2404 ± 120 TEAC), ABTS (8694 ± 435 TEAC) and FRAP (581 ± 29 de AEAC) (Montoya et al., 2012). The antioxidant activities of phenolic compounds are mainly contributed to their redox properties, allowing them to act as reducing agents, hydrogen donors and single oxygen quenchers (Ljevar et al., 2016).

A diet rich in antioxidant might prevent different some non-communicable chronic diseases such as cancer, cardiovascular and neurodegenerative diseases (Wiczkowski et al., 2014; Pace et al., 2014). A big number of studies suggest that wine consumption might reduce the risk of heart attack, and give anti-inflamatory, anti-carcinogenic, anti-viral, anti-bacterial effects (King et al., 2006; Apostolidou et al., 2015). Nevertheless, the antioxidant value from foods can be lost easily according to the food treatment.

An alternative to preserve the antioxidant value of Andean berry is the alcoholic beverage production. Different authors suggest that after a fermentation process, the antioxidant value of fruit still remains in wine and can be preserved during long periods of time (Rai and Anu Appaiah, 2014). However, different maceration methods can affect the antioxidant power in wine. The most common methods of maceration are: 1- Mechanical maceration, 2- fruit preheating, 3- enzymatic hydrolysis and 4- fruit filtration under pressure (Duarte et al., 2010; Ahmed, 2011; Ubeda et al., 2013).

Due to the previous mentioned, this study proposes the following objectives (i) to produce three types of alcoholic beverages from Andean berry, with three different maceration methods; preheating, mechanical maceration and a combination of both, (ii) to measure the antioxidant activity and (iii) to determinate anti-proliferative activity.

MATERIALS AND METHODS

Fruits

Andean berry (*V. meridionale Sw*) were harvested in Santa Elena village, located in the rural area of Medellin city, at 2600 m.a.s.l, with an average temperature of 14.5°C and relative humidity of 89%. This material had a voucher number ILS 14050070.

Treatment of must

Must were prepared adding 1 kg of fruit and 1 L of water according to modified methods proposed by of Solieri and Giudici (2008) and Ferreira et al. (2009). To obtain the beverages three different methods were used, macerated fruit machine (MAC), preheating of the fruit (CAL) at 80°C during 15 min and a combination of both of them (MIX).

Yeast and inoculation

The yeast was prepared according to the procedure described by Coronel (2008) with some modifications. *Saccharomyces cerevisae* (Pasteur Red Star), 0.4 g of yeast per 100 mL of beverage was used. It was activated in water at 37°C during 10 min.

Antioxidant activity

DPPH method

Radical scavenging activity against the stable radical DPPH was measured using the methods proposed by 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. The absorbance decrease, associated with a reduction in the DPPH concentration, was measured at 517 nm. The results were expressed in trolox equivalents antioxidant capacity (TEAC).

FRAP assay

The antioxidant capacity of wine was estimated according to the procedure described by Benzie and Strain (1996), with some modifications. This method is based on the increase of absorbance due to the formation of 2, 4, 6-tripyridil-s-triazine (TPTZ)-Fe (II) in the presence of reducing agents. A volume of 50 µl of extract was mixed with 950 µl FRAP reagent previously 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 L) using an ascorbic acid standard curve.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC Essay was determined using the following methodology: 3 mL were prepared with 21 µl of 10 µM fluorescein solution, 2899 µl of 75 µM 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 spectrofluorometer with a thermostated multicell. The ORAC value µmol Trolox/L was calculated by using the following equation:

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

Where AUC is THE area under THE curve FOR sample CONTROL (AUC°), and trolox reference (AUC_{Trolox}) (Zapata et al., 2013).

Phenolic content

Total anthocyanins

Anthocyanins were determined by pH differencial method. The absorbance was measured at 530 nm and 700 nm in buffers of 1 and 4.5 pH. The expression A = [(A530 – A700) pH1.0 – (A530 – A700) pH 4.5] was used for calculating anthocyanins. Their value was expressed as Cianidine-3-glucoside/L (Zambrano-Moreno et al., 2015).

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

Table 1. Ethanol concentration, yield and productivity of Andean berry wine.

Treatment	Ethanol (g/L)	Yield (Yp/s) g/g	Productivity (P) g/day L
MAC	90 ± 1.7[a]	0.36 ± 0.01[a]	5.04 ± 0.09[a]
MIX	94 ± 4.1[a]	0.38 ± 0.02[a]	5.28 ± 0.23[b]
CAL	89 ± 3.6[a]	0.36 ± 0.01[a]	4.94 ± 0.20[a]

Values are mean ± SD (n=4), value not sharing common alphabets for the same attribute are significantly different (P<0.05).

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 control sample. Aqueous solutions of gallic acid were used to build a calibration curve. The results were expressed as gallic acid equivalents (GAE)/L (Zapata et al., 2013).

Hydroxycinnamic acids determination by HPLC–DAD

Hydroxycinnamic acids were analyzed by direct injection of previously filtered samples through a 0.45 Rm 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) C18 column (5 Rm) 250 × 4.6 mm (Restek©, Bellefonte, USA) with an auto injector 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).

Cell culture

SW480 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured according to a procedure previously described (Maldonado et al., 2014). Cells were cultured in 75 cm^2 Falcon tubes with modified eagle´s medium (Dulbecco), supplemented with 25 mM glucose, 2 mM L-glutamine, 10% inactivated horse serum (heated at 56°C), 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% of CO_2. 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 during 24 h after seeding, to different concentration of fermented beverage which etanol was eliminated through rotative evaporation at 30°C under vacuum.

Sulforhodamina B (SRB) assay

The effect of extracts on growth cells were 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. 3000 viable cells from each cell line were exposed to extracts during 24 h after being seeded and incubated for different times. Control cells were treated with 0.1% dimethyl sulphoxide (DMSO). Culture media was replaced every 48 h. The cell culture growing 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 2×10^5 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 is the control (non-treated cells).

Statistical analysis

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

RESULTS

Ethanol concentration, yield and productivity of fermented Andean berry beverages are reported in Table 1. The differences in ethanol concentration and yield were not statistically significant ($p < 0.05$) in fermented beverage.

Antioxidant activity and phenolic compounds of must and fermented beverages of Andean berry (wine)

Three treatments (MAC, CAL and MIX) for getting musts were carried out. They were analyzed by DPPH, FRAP and ORAC techniques in order to measure antioxidant power. Totals phenols and anthocyanins were also quantified. Results for the above treatments showed statistical differences and they are represented in Figure 1. Results suggest that heated treatments MIX and CAL increased the extraction of phenolic metabolites and the antioxidant power. On the contrary, CAL treatment did not produce the same effect and results were lower. MIX and CAL did not show any significant differences between them, regarding to antioxidant activity and phenolic compounds.

Fermented beverages were also analyzed by using the same techniques. CAL and MIX fermented treatments presented higher values in DPPH, total phenols and

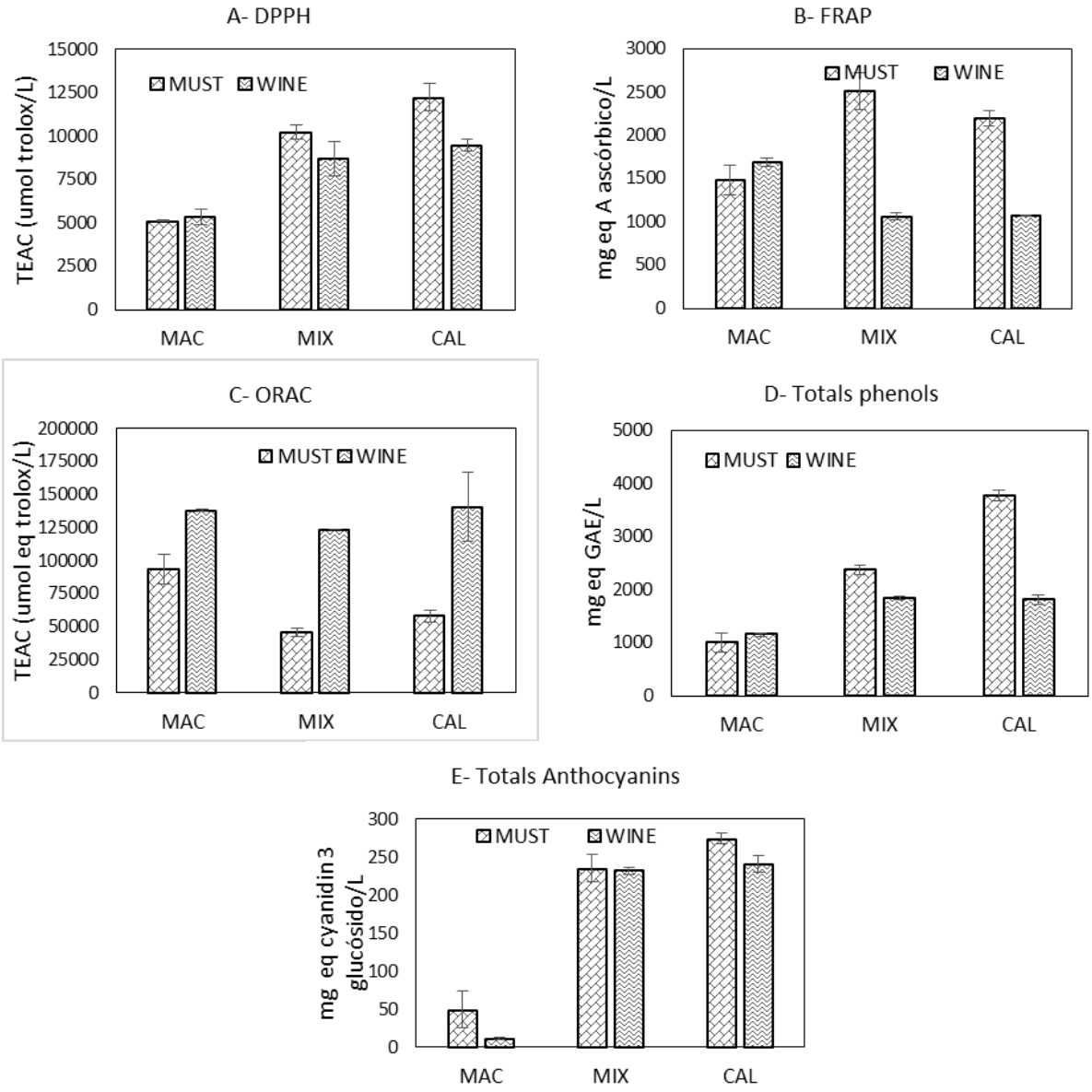

Figure 1. Comparison of antioxidant activity A) DPPH, B) FRAP y C) ORAC, and secondary metabolites content D) Total phenols and E) Total anthocyanins of must and fermented beverages from Andean berry.

anthocyanins assays. While MAC fermented treatment showed higher values in the FRAP assay. Oxygen radical absorbance capacity (ORAC) assay did not exhibit significant statistical differences between the three fermented treatments.

The must and its fermented beverages were compared. Findings indicate that the fermentation process contributed to decrease the totals phenols and the antioxidant activity which was measured by FRAP and DPPH for MIX and CAL treatments.

According to the anthocyanins, they were preserved in the same treatments after the fermentation process (CAL, MAC, MIX). However, the antioxidant activity measured by ORAC increased for the three treatments. Finally, MAC treatment preserved totals phenols content and the antioxidant power, this last one was measured by FRAP and DPPH. Hydroxycinnamic acid results are reported in Table 2. MAC, MIX and CAL treatments showed a higher value in chlorogenic acid, p-coumaric acid and ferulic acid respectively.

Antiproliferative activity of CAL fermented beverages

The pharmaceutical function of fermented beverages was tested. The effect of fermented beverages on SW480 cell

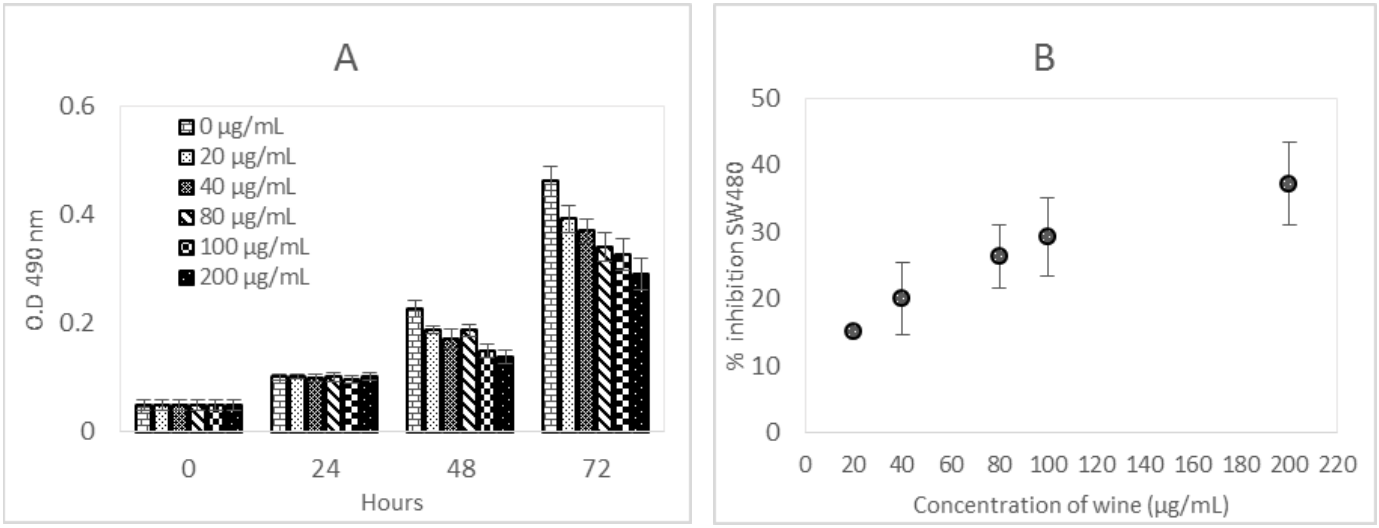

Figure 2. A) Effect of dealcoholized Andean berry wine on SW480 cell growth. B) % Inhibition vs concentration of dealcoholized wine.

Table 2. Hydroxycinnamic acid in fermented beverages of Andean berry.

Tratamiento	Chlorogenic acid (mg/L)	P-Coumaric acid (mg/L)	Ferulic acid (mg/L)
MAC	3.21 ± 0.29[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
MIX	1.10 ± 0.03[b]	2.36 ± 0.12[b]	0.00 ± 0.00[a]
CAL	0.47 ± 0.06[c]	0.21 ± 0.00[c]	2.67 ± 0.03[b]

Values are mean ± SD (n=4), value not sharing common alphabets for the same attribute are significantly different (P<0.05).

growth is represented in Figure 2A as OD of cell proteins at 490 nm treated or not with the extract at different concentrations (0 to 200 µg/mL). The OD of SW480 cell protein was reduced 37.3% at 200 µg/mL (Figure 2B). The inhibitory effect on cell viability on SW480 increased significantly ($p < 0.05$) as concentration increased from 20 to 200 µg/mL. The IC_{50} value calculated from the non-linear regression between percent of inhibition and logarithm concentration was found SW480: IC_{50} = 139.1 µg/mL.

DISCUSSION

Results suggest that by using this methodology is not possible to obtain industrial yields. Experimental yield can vary from 90 – 95% of theoretical yield. However, in the industry, this yield can vary between 87 and 93% (Vasquez and Dacosta, 2007). In this assay we obtained 70.5% (MAC and CAL) and 74.5% (MIX) of yield. This lower value might be caused by yeast using glucose to produce other metabolites or adapting to the substratum.

Heating treatments presented better results over mechanical treatment due to the high temperature. This aided the extraction of phenolic compounds thanks to the solubility effect of metabolites and diffusion rate of these compounds in solution. According to Suteerapataranon et al. (2009), the solubility and diffusion coefficient increase when temperature increase. Moreover, thermal treatment may concentrate the compounds of fruits by evaporation (López de Lerma and Peinado, 2011; Torija et al., 2003). Other authors also report that after pasteurization reducing sugar, soluble solids, acidity and flavonoids might be increased (Nurgel et al., 2002; Ferreira et al., 2009).

MAC treatment presented a lower value of antioxidant activity. It suggests that the medium may be oxidized by polyphenol oxidase enzyme. The mechanical maceration increases the area of contact of oxygen which improves enzymatic action. As the opposite, the enzyme is inhibited by heating in CAL and MIX treatments.

Our results are similar to those reported by Porgali et al. (2012), according to the analysis of red wines antioxidant activity. This author reported a value of 1836 ± 40.5 mg GAE eq/L for Karpat wine and 3466 ± 54.4 mg GAE eq/L for Buzbag wine in totals phenols. Besides, Granato et al. (2010) reported a range of 1041.63 – 1958.78 mg GAE/L for Brazilian red wines. And Baroni et al. (2012), reported values of 3.6 ± 2.8 mg/L ferulic acid for Cabernet Sauvignon wine, 4.1 ± 2.7 mg/L for Malbec

and 2.9 ± 3.2 mg/L for Shyrah. Additionally, this author reported a Coumaric acid value of 4.5 ± 1.2, 7.3 ± 2.1 and 5.7 ± 5.2 mg/L for Cabernet Sauvignon, Malbec and Shyrah wine respectively.

Statistical analysis indicates that there is an influence of pretreatment method of fruit on the antioxidant power of fermented beverage ($P<0.05$). CAL and MIX treatments presented a higher value in antioxidant power. According to this, Granato et al. (2011) and Ubeda et al. (2013) reported that antioxidant activity is affected by different factors such as grapes varieties, different maturities, maceration mechanism, and temperature of maceration.

Rai and Anu Appaiah (2014) demonstrated that totals phenols concentration may increase or decrease during fermentation, depending on process conditions. Other authors reported that free radical scavenger capacity (DPPH), anthocyanins and flavonoids are reduced during fermentation (Pérez-Gregorio et al., 2011). Those findings are similar to the results reported in this study, where DPPH and totals phenols were reduced during fermentation in MAC and CAL treatments.

On the other hand, extreme temperatures can generate labile anthocyanins. In the presence of oxygen, methoxyl, glucosyl and acyl substitutions increase causing a rise in the pH where the maximum thermal stability occurs (Jackman and Smith, 1996). It influences the rate and the mechanism of anthocyanin thermal degradation (Hazdrina et al., 1970).

Dealcoholized fermented beverage of Andean berry was cytotoxic in a dose dependent manner with a maximum effect at 200 µg/mL. This fermented beverage contains a range of biologically active metabolites that showed being a potent anticancer. Anthocyanins might provide a lot of effects such as the reactive oxygen species scavenger capacity, chelate metals, stimulating the expression of enzymes, reducing the formation of oxidative DNA adduct, reducing lipid peroxidation inhibiting toxins and environmental mutagenesis carcinogens, and reducing cell proliferation by modulating the signal transduction pathways (Wang et al., 2000; Wang et al., 2008). Hidroxycinamic acids have been also associated to antiproliferative effect (Yi et al., 2005). Other authors have reported a constantly increased of cytotoxicity up to 61 and 78% at 100 and 200 µg/mL GAE, respectively, after 48 h for lyophilized Trigaio red wine in osteosarcoma cell line (Tedesco et al., 2013; Oliveira et al., 2015).

The IC50 value for this assay was 139.1 µg/mL which according to Criteria of national cancer institute of USA; is considerate active when IC50 value is less than 30 µg/mL in cancer cells lines (Suffnes and Pezzutto, 1990). According to the above mentioned, this beverage shows low cytotoxic activity in spite of high content of total anthocyanins preserved after CAL fermentation beverage. The results obtained here suggest that other phenol compounds different to anthocyanins such as phenolic acids (hydroxycinamic acid, p-coumaric acid) whose concentration was significantly decreased, might be required to induce an important antiproliferative activity. Further analyses will be required to answer this question.

Conclusion

Yield and productivity parameters were independent of fruit macerated treatment. Antioxidant activity and totals phenols were higher in heating treatments (CAL and MIX) due to the temperature which increased the diffusion coefficient of secondary metabolites and inhibiting some oxidative enzymes. During fermentation, antioxidant activity was decreased for CAL and MIX treatments, but for MAC treatment, IT was preserved. The dealcoholized fermented beverage decreased the viability of colon cancer cells. However, according to criteria of national cancer institute of USA, this beverage presents low antiproliferative activity.

Finally, this research suggests that the dealcoholized fermented beverages can become an interesting strategy in cancer chemoprevention tool in secondary prevention of cancer or in adjuvant chemotherapy owing to metabolites contents and antioxidant power.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

To the Estrategia de Sostenibilidad 2014 – 2015 de la Universidad de Antioquia.

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academicJournals

Vol. 10(27), pp. 409-416, 17 July, 2016
DOI: 10.5897/JMPR2015.5979
Article Number: F679AE659546
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Phytochemical screening, total phenolic content and antioxidant activity of some plants from Brazilian flora

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Received 19 October 2015; Accepted 4 April, 2016

The present study evaluated the total phenolic and flavonoid content as well as the antioxidant activity of methanolic leaf extracts of five plants from Brazilian flora: *Abarema cochliacarpos*, *Croton corchoropsis*, *Myroxylon peruiferum*, *Stryphnodendron pulcherrimum* and *Tanaecium cyrtanthum* by 2,2'-azino-bis- (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and total antioxidant capacity assays. A thin layer chromatography analysis of all plant extracts has also been performed and it showed the presence of different types of secondary metabolites, namely saponins, phenylpropanoids and flavonoids. Among the studied plants, *A. cochliacarpos* and *S. pulcherrimum* showed considerable antioxidant radical scavenging activity on all the tested assays and they also exhibited substantial amounts of phenolic compounds. In addition, a positive correlation was found between total phenols and both ABTS radical scavenging activity and total antioxidant capacity assays, thus indicating the major role of phenols on the antioxidant activity of these plants. To the best of the authors' knowledge, this is the first approach where the phenolic content and antioxidant activity of *A. cochliacarpos, C. corchoropsis, M. peruiferum, S. pulcherrimum* and *T. xanthophyllum* were explored.

Key words: Brazilian medicinal plants, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), flavonoid content, phenolic content.

INTRODUCTION

Oxidation and reduction of molecules are essential to life; they represent normal phenomena that occur in cell metabolism. Among substances involved in oxidation-reduction reactions of molecules are free radicals, which are organic or inorganic compounds having one or more unpaired electrons on their valance shell, they are chemically unstable and very reactive (Lushchak, 2014).

In organism, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in metabolic processes such as energy production, regulation of cell

growth, intercellular signaling, phagocytosis and synthesis of important biological molecules. For many years, chemists have known that free radicals cause oxidation, which can be controlled or prevented by a range of antioxidants substances (Bild et al., 2013; Rahal et al., 2014).

The amount of free radicals in the body is counterbalanced by the availability of antioxidants, which are compounds capable of either preventing formation of free radicals or by reacting with them directly. The imbalance of free radicals/antioxidants in favor of free radicals can lead to establishment of oxidative stress, a situation characterized by biomolecule impairment and consequently to human health peril (Rajendran et al., 2014).

Antioxidants are believed to play an important role in the prevention of several diseases (Ngo et al., 2011). The therapeutic effects of various natural plant-derived medicines are correlated with their antioxidant activity (Ezhilarasan et al., 2014). Among molecules produced by plants, the polyphenols are one of the most widely studied classes whose remarkable antioxidant capacity are credited primarily due to their reducing properties and chemical structure (Barreiros et al., 2006). Studies indicate that consumption of fruits and vegetables containing phenolic antioxidants and other phytochemicals is advantageous for health (Almeida et al., 2011).

Antioxidants are applied in food industry as agents that prevent autoxidation of meat, fruit and oils, where several compounds such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butyl hydroquinone (TBHQ) are produced for this purpose (Karpińska-Tymoszczyk, 2014). However, there is growing concern over the possible carcinogenic effects of synthetic antioxidants in foods (Juntachote et al., 2006). Yet, the potential health risks by the use of synthetic antioxidants have triggered the interest in the search for natural antioxidants (Mohamed et al., 2011).

Abarema cochliocarpos (Gomes) Barneby & Grimes (Fabaceae) is a plant popularly known as "barbatimão", "bordão-de-velho", "ingá-negro" and "barbimão" (Iganci and Morin, 2012). This plant is mainly found in the Brazilian Atlantic Forest and Caatinga biomes (Watkinson, 2012). Regarding the biological activities of this species, antimicrobial, antinociceptive, healing and antioxidant properties have been described (Santos et al., 2007; Silva et al., 2009; Sánchez-Fidalgo et al., 2013). The decoction from its bark is utilized in folk medicine as wound-healing, antiseptic, analgesic and it is used against dermatosis, leukorrhea, inflammation and gastric ulcer (Araújo et al., 2002; Santos et al., 2007; Silva et al., 2010a).

Myroxylon peruiferum L. f. (Fabaceae) is a tree present all over the Brazilian territory, especially at semideciduous forests and it is locally known as “cabreúva” (Sebbenn et al., 1998). Ohsaki et al. (1999) evaluated its antimicrobial potential against *Helicobacter pylori*, but studies on the biological activity for this species are scarce. From its wood can be extracted an exudate known as “balsam of Peru” or “Tolu”, it is used in folk medicine against coughs, bronchitis, diabetes and sedative in case of urinary problems (Rizzini and Mors, 1995).

Croton corchoropsis Baill (Euphorbiaceae) is a subshrub plant found in Brazilian Cerrado, Campos Rupestres and Caatinga biomes (Silva J et al., 2009). Although, some studies involving biological activities of plants of this genus have been reported in the literature (Morais et al., 2006; Salatino et al., 2007), so far, no studies of antioxidant activity for *C. corchoropsis* were performed. *Stryphnodendron pulcherrimum* Mart. (Fabaceae) is an arboreal species occurring in the Amazonian forest and Atlantic forest in northeast Brazil (Scalon, 2007). Castilho et al. (2013) reported the antibacterial activity of extracts of this plant against oral pathogens.

Tanaecium cyrtanthum (Mart. ex DC.) Bureau & K.Schum. (Bignoniaceae) is a liana that can be found in northeast Brazil (Lohmann and Taylor, 2014). Studies regarding this species are rare. All plants listed in this study are species found in the Caatinga biome, a semi-arid climate region which offers a set of characteristic environment conditions that are believed to promote an augmentation in plant production of secondary metabolites, some of those molecules in turn can have antioxidant properties (Lemos and Zappi, 2012; Chaves et al., 2013; Alamgir et al., 2014).

All these plant species are found in Pernambuco state (northeast Brazil) inside rural community regions where locals make use of folk medicine as their primary healthcare, but studies are lacking to give support for these traditional plant usages as well to promote proper exploitation of new phytotherapics. Overall, *M peruiferum*, *C. corchoropsis*, *S. pulcherrimum* and *T. cyrtanthum* are poorly studied plants and with the exception of *M. peruiferum*, so far no articles were found on leaf extracts of these species.

This study aimed to conduct analyses of antioxidant activity as well as the total phenol and flavonoid contents in methanolic leaf extracts of five plants (*A. cochliacarpos*, *C. corchoropsis*, *M. peruiferum*, *S. pulcherrimum* and *T. cyrtanthum*) located in Pernambuco state, Brazil and to correlate the total phenolic and

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flavonoid content with the antioxidant activities of the extracts.

MATERIALS AND METHODS

Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis- (3-ethyl benzothiazoline-6-sulfonate) (ABTS), gallic acid, Folin-ciocalteu's reagent, quercetin and trolox were purchased from Sigma-Aldrich. Sodium phosphate, ammonium molybdate were from Vetec (Brazil); ascorbic acid from Anidrol (Brazil), sulfuric acid from Cinética (Brazil) and potassium persulfate from Labsynth (Brazil). All the chemicals including solvents were of analytical grade.

Plant material

The plants chosen for this study were based on ethnobotanical data available in the literature (Agra et al., 2007, 2008). The plant species, *A. cochliacarpos* (Gomes) Barneby & Grimes (Fabaceae) and *S. pulcherrimum* (Willd.) Hochr. (Fabaceae), were collected at Reserva de Floresta Urbana Mata de Camaçari, Cabo de Santo Agostinho, Pernambuco State, Brazil, a Conservation Unit of the Atlantic Forest biome. *C. corchoropsis* Baill. (Euphorbiaceae), *M. peruiferum* L.f. (Fabaceae) and *T. cyrtanthum* (Mart. ex DC.) Bureau & K.Schum. (Bignoniaceae) were collected at Parque Nacional do Catimbau, Conservation Unit of the Caatinga biome, Municipalities of Buíque, Ibimirim and Tupanatinga, Pernambuco State, Brazil. Leaves were collected in February (2012) and the plants were botanically identified at Herbarium IPA, from Instituto Agronômico de Pernambuco (Agronomic Institut of Pernambuco State), Brazil. Vouchers species: *A. cochliacarpos* (92001), *S. pulcherrimum* (92002), *C. corchoropsis* (92003), *M. peruiferum* (92004) and *T. cyrtanthum* (92005) were deposited at the herbarium IPA.

Preparation of leaf extracts

The leaves were dried in forced-air circulation oven at 40°C for 72 h, pulverized with a grinder, and stored at room temperature in closed containers until use. The powdered leaves of the five species (100 g, each) were extracted in Soxhlet extractor at 65°C containing 1000 mL of methanol. The material was refluxed for about 48 h until exhaustion and the resulting extracts were filtrated through Whatman No.4 filter paper. The extracts were then concentrated in vacuum at 40 °C using a Rotary Evaporator. Then the extracts were preserved in sealed vials at 4°C until further analyses.

Phytochemical screening

The phytochemical screening of the extracts was performed by thin-layer chromatography (TLC) on silica plates (60F254, aluminum backed, 200 µm layer thickness, 10.0 x 5.0 cm, Macherey-Nagel, Ref. 818160, Germany). The presence of flavonoids, phenylpropanoids, alkaloids, terpenes, steroids, coumarins, quinones and proanthocyanidins were investigated using adequate development systems and revealers, as shown in Table 1 (Roberts et al., 1957; Brasseur and Angenot, 1986; Wagner and Bladt, 1996; Harborne, 1998). After development, the plates were air dried and sprayed with the revealers in a fume hood.

Estimation of total phenolic content

The total phenolic content was determined by Folin-Ciocalteu method (Singleton and Rossi, 1965) with minor modifications. 200 µL aliquots of plant extract s at 1 mg/mL were mixed with 1 mL of Folin Ciocalteu reagent (1: 1 v/v) and 2.5 mL of 20% Na_2CO_3 were added. The mixtures were incubated for 30 min at room temperature and protected from light for subsequent reading of absorbance against a blank solution consisting of methanol plus all reagents without extracts. They were read in a 765 nm spectrophotometer and the total phenolic content was calculated using gallic acid as reference in the range of 25-500 mg/mL. The results were expressed in mg of gallic acid equivalents per extract gram (mgGAE.g^{-1} extract).

Estimation of total flavonoid content

The flavonoid contents were measured by aluminum chloride colorimetric method (Woisky and Salatino, 1998). 500 µL of samples (1 mg/mL) were added to 500 µL of 2% methanolic $AlCl_3$. After 1 h incubation at room temperature, the absorbance was measured against a blank of methanol and aluminum chloride in a spectrophotometer at 420 nm. Flavonoid content was estimated using a quercetin standard curve (0.98-7.81 µg/mL) and the results were expressed as mg of quercetin equivalent per extract gram (mg QE. g^{-1} extract).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The DPPH free radical scavenging activity of the extracts was performed according to Brand-Williams et al. (1995) with some modifications. A stock solution of DPPH 200 µM in methanol was further diluted in methanol to obtain an absorbance between 0.6 - 0.7 at 517 nm, resulting in the DPPH working solution. Different concentrations of the extracts were mixed with DPPH solution and after 30 min incubation in darkness, the absorbance were read at the same wavelength mentioned above. Then it was plotted a graph of DPPH scavenging activity against different concentrations of extracts to calculate the IC_{50}, which denotes the sample concentration required to decrease the initial DPPH radical concentration by 50%. Gallic acid was used as standard. The measurements were triplicate and their scavenging activities were calculated based on the percentage of DPPH scavenged.

2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging assay

ABTS radical scavenging assay was carried out according to Re et al. (1999) with minor modifications. Briefly, a radical ABTS stock solution was prepared by dissolving ABTS (7 mM) with potassium persulphate ($K_2S_2O_8$, 2.45 mM). The mixture was left to stand for 16 h (time required for the formation of the radical) in the dark at room temperature before use. To perform the assay, the previously made solution was diluted in ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm, thereby obtaining the ABTS radical working solution. 30 µL plant extracts (1 mg/mL) were mixed with the working solution and left to rest for 6 min before measuring the absorbance at 734 nm against a blank (working solution plus methanol) and it was applied as standard.

Evaluation of total antioxidant capacity

The total antioxidant capacity (TAC) was based on the method of Prieto et al. (1999). 0.1 mL of the extracts (1 mg/mL) were combined with 1 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the solutions were incubated at 95°C for 90 min, then cooled to room temperature and the absorbance of each

Table 1. Development systems and revealers used for phytochemical screening by thin-layer chromatography.

Secondary metabolites	Standard	Development system	Revealer
Flavonoids and phenylpropanoids	Quercetin, rutin and chlorogenic acid	EtOAc-HCOOH-AcOH-H_2O (100:11:11:27 v/v)	Neu's reagent
Alkaloids	Pilocarpine	EtOAc-HCOOH-AcOH-H_2O (100:11:11:27 v/v)	Dragendorff's reagent
Triterpenes and steroids	β-sitosterol and ursolic acid	Toluol:EtOAc (90:12 v/v)	Liebermann-Burchard's reagent
Mono and sesquiterpenes	Thymol	Toluol:EtOAc (97:03 v/v)	Anisaldehyde-sulphuric acid reagent
Coumarins and quinones	Coumarin and lapachol	$CHCl_3$-MeOH (98:2 v/v)	KOH
Proanthocyanidins	Catequin	EtOAc-HCOOH-AcOH-H_2O (100:11:11:27 v/v)	Vanillin- hydrochloric acid reagent

Table 2. Phytochemical screening of plant extracts by thin layer chromatography.

Secondary metabolites	*Abarema cochliacarpos*	*Croton corchoropsis*	*Myroxylum peruiferum*	*Stryphnodendron pulcherrimum*	*Tanaecium cyrtanthum*
Saponins	+++	+	+++	+++	+
Flavonoids	++	+	+	+	+
Phenylpropanoids	++	++	+	+	+
Triterpenes and steroids	*	-	-	-	-
Mono and sesquiterpenes	+	-	*	*	-
Alkaloids	-	+	-	-	-
Proanthocyanidins	+ [(1)]	-	-	+ [(1)]	-
Coumarins	-	-	+	-	-
Quinones	-	-	-	-	-

[1]Polymeric proanthocyanidins, monomeric and dimeric proanthocyanidins absent; *only traces detectable.

sample were measured at 695 nm against a blank (1 mL of reagent plus 0.1 mL of methanol).

Statistical analysis

Assays were performed in triplicate and th results are shown as mean ± standard deviation. Linear regression analysis and Pearson's correlation coefficient were calculated using Microsoft Excel Windows 2013.

RESULTS AND DISCUSSION

Phytochemical screening by TLC indicated the presence of different types of secondary metabolites, namely flavonoids, saponins and phenylpropanoids in all plant extracts. *A. cochliacarpos* and *S. pulcherrimum* also showed the presence of polymeric proanthocyanidins, while *M. peruiferum* showed the presence of coumarins. Alkaloids were detected only in *C. corchoropsis* (Table 2). Preliminary phytochemical screening experiments are commonly performed to promote a guidance of substantial phytochemicals that may be involved in the antioxidant activity of plant extracts (Anandakirouchenane et al., 2013; Basma et al., 2011; Das et al., 2012).

Phenolic compounds are considered important natural antioxidants and represent one of the most abundant compounds in plants. They display several functions such as pigmentation, protection against ultraviolet rays, allelopathic action, defense against microbial attack and predators (Naczk and Shahidi, 2006). The total phenolic content of the extracts ranged from 28.84 to 120.39 $mgGAE.g^{-1}$, it was higher for *A. cochliacarpos* (120.39 ± 2.82 $mgGAE.g^{-1}$), followed by *S. pulcherrimum* (86.67 ± 0.83 $mgGAE.g^{-1}$) (Table 3).

Phenolic compounds exhibit their antioxidant activity by various mechanisms such as donation of hydrogen atoms to free radicals and through connection to transition metal ions resulting in more stable forms (Kumar et al., 2014). Various physiological actions performed by polyphenols were related to the prevention of neurodegenerative and cardiovascular diseases, cancer, among others, mainly because of their high antioxidant capacity (Wootton-Beard et al., 2011).

Table 3. Total phenolic content (mgGAE.g^{-1}) and flavonoid content (mgQE.g^{-1}).

Plant extracts	Phenolic (mgGAE.g^{-1})	Flavonoid (mgQE.g^{-1})
Abarema cochliacarpos	120.39 ± 2.82	5.55 ± 0.13
Stryphnodendron pulcherrimum	86.67 ± 0.84	6.04 ± 0.18
Myroxylum peruiferum	42.18 ± 3.57	10.52 ± 0.55
Tanaecium cyrtanthum	30.16 ± 1.41	3.84 ± 0.03
Croton corchoropsis	28.84 ± 1.17	4.11 ± 0.14

GAE: Gallic acid equivalent; QE: quercetin equivalent.

Table 4. Effect of methanolic extracts on different antioxidant models.

Plant extracts	DPPH IC_{50}	$ABTS^{+}$ (%)	TAC (%)
Abarema cochliacarpos	31.62 ± 2.28	75.69 ± 3.88	43.71 ± 1.66
Stryphnodendron pulcherrimum	35.47 ± 2.48	69.65 ± 5.60	41.91 ± 1.91
Myroxylum peruiferum	37.26 ± 1.54	20.92 ± 3.41	15.55 ± 0.87
Tanaecium cyrtanthum	43.77 ± 2.10	30.83 ± 2.65	21.75 ± 1.66
Croton corchoropsis	87.84 ± 1.74	33.66 ± 1.67	15.13 ± 1.52

Data are expressed as means of three replicates.

Among the polyphenol compounds, the most studied subclass is the flavonoids which in plants are commonly found conjugated to sugars (Wang et al., 2013). The total flavonoid content was quantified by the aluminum chloride method and expressed as quercetin equivalents (QE) per gram of substrate. The total flavonoid content was in the range of 3.84 to 10.52 mg QE.g^{-1} in which *M. peruiferum* had the highest concentration among the five extracts (10.51 ± 0.55 mg QE.g^{-1}) (Table 3).

Silva et al. (2010b) reported the occurrence of polyphenolic compounds highlighting catechins (flavonoids) the major constituent in butanolic extracts of *Abarema cochliocarpos* bark. Mathias et al. (2000) reported the presence of isoflavones, pterocarpans, coumestans, flavanone and isoflavanones from the bark of *Myroxylon peruiferum* and Carvalho et al. (2008) isolated 3 compounds from its leaves, namely 3',4',7-trimethoxyisoflavone (cabreuvina), 6-hydroxy-4',7-dimethoxyisoflavone and germacrene D. Some species of *Stryphnodendron* genus are known for their high polyphenol content of tannins (Lima et al., 2010). Phenolic substances have been reported for several *Croton* species, among which are flavonoids, lignoids and proanthocyanidins predominate (Salatino et al., 2007). As for *Tanaecium cyrtanthum*, there are only few reports in the literature on this species and no phytochemical assessment was found.

The antioxidant activity of the extracts by DPPH, ABTS and total antioxidant capacity (TAC) methods are outlined in Table 4. IC_{50} values for DPPH scavenging activity were smaller for *A. cochliocarpos* (31.62 ± 2.28 µg/mL) and *S. pulcherrimum* (35.47 ± 2.48 µg/mL) followed by *M. peruiferum* (37.26 ± 1.54 µg/mL) (Table 4). The lower IC_{50} value indicates higher antioxidant capacity, therefore *A. cochliacarpus* and *S. pulcherrimum* showed the best results. The ABTS radical scavenging activity was higher for *A. cochliocarpos* (75.69 ± 3.88%); followed by *S. pulcherrimum* (69.65 ± 5.60%) (Table 4). *A. cochliocarpos* and *S. pulcherrimum* extracts obtained the highest antioxidant activities in both DPPH and ABTS assays.

DPPH and ABTS radical scavenging assays are methods based on the sequestration of these radicals by proton donor substance, the reaction is followed by a measurable change in absorbance spectrophotometry (Floegel et al., 2011). Both DPPH and ABTS assays are widely used to assess the antioxidant capacity of natural products (Schaich et al., 2015).

The total antioxidant capacity (TAC) is a spectrophotometric assay based on the reduction of Mo(VI) to Mo(V) by the action of an antioxidant substance with the subsequent formation of a green phosphate/Mo(V) complex with a maximum absorption at 695 nm (Prieto et al., 1999). The total antioxidant capacity (%TAC) of the extracts were higher in *A. cochliocarpos* (43.71 ± 1.66%) and *S. pulcherrimum* (41.91 ± 1.91%) (Table 4). In the same manner of DPPH and ABTS assays, *A. cochliocarpos* and *S. pulcherrimum*

Table 5. Correlation between total phenolic content, total flavonoid content and antioxidant assays

Parameter	Total phenolic content		Total flavonoid content	
	R^2	ρ	R^2	ρ
DPPH $1/IC_{50}$	0.564	0.751	0.182	0.427
$ABTS^+$ (%)	0.858	0.926	0.068	-0.261
TAC (%)	0.872	0.933	0.026	-0.163

R^2: Coefficient of determination, ρ: Pearson coefficient.

had the highest total antioxidant activity, in addition they also were the ones that showed the highest total phenolic content among the studied plants (Table 3). It is worthy to note that the possible antioxidant potentials of plant extracts commonly depend on the phytochemical composition and extraction systems including methods, duration and polarity of organic solvents. For this reason, the antioxidant potentials cannot be completely described using one single method. In this context, more than one antioxidant test system is required to determine the mechanisms of antioxidant actions of plant extracts (Wong et al., 2006).

The total phenolic and flavonoid contents of the extracts were compared with the values of their respective antioxidant activities by using the Pearson coefficient (ρ) and coefficient of determination (R^2), where a positive correlation can be found between total phenols and ABTS radical scavenging activity (ρ = 0.926, R^2 = 0.858) as well as phenolic compounds and TAC (ρ = 0.933, R^2 = 0.872) (Table 5). Whereas flavonoid content of the extracts showed no significant correlation with the antioxidant activities (Table 5). These results suggest the importance of phenolic compounds (not flavonoids) on the antioxidant activity (%$ABTS^+$ and %TAC assays) of these plant extracts, however we cannot neglect the potential influence of other bioactive molecules that may be included in the extracts such as tocopherols, saponins, polysaccharides and ascorbic acid (Ananthi et al., 2010).

Kumar et al. (2014) using methanolic extracts of *Lantana camara* L. leaves obtained similar results when comparing phenolic content with ABTS activity (ρ = 0.998, R^2 = 0.997) and TAC (ρ = 0.946, R^2 = 0.896). In the same way, Basma et al. (2011) assessing antioxidant activities of *Euphorbia hirta* extracts found a relevant correlation between phenol content and IC_{50} DPPH values (R^2 = 0.989) but on the other hand, a moderate correlation was found between flavonoid content and IC_{50} DPPH (R^2 = 0.696). Silva et al. (2011) working with hydroalcoholic extracts of *Anadenanthera colubrina*, *Libidibia ferrea* and *Pityrocarpa moniliformis* fruits noted correlation between polyphenols and TAC (ρ = 0.923, R^2 = 0.862). In fact it is well known that there is a strong relationship between phenolic content and antioxidant activity in plants (Abdelhady et al., 2011; Hossain and Shah, 2015).

Conclusions

Based on the results obtained in the present study, it is concluded that the methanolic leaf extracts of *A. cochliacarpos* and *S. pulcherrimum* exhibit considerable antioxidant radical scavenging activity on all tested assays and they possess substantial amounts of phenolic compounds. Thus, these 2 plants can be considered a good source of antioxidants which might be beneficial for combating oxidative stress. A positive correlation was found between total phenolic content and ABTS radical scavenging activity as well as total antioxidant capacity assays of the plant extracts, thus indicating the key role that phenolic compounds may exert on the antioxidant activity of these plants. Hence more studies are required to isolate and identify these bioactive compounds responsible for such activities so as to assess their antioxidant activity *in vivo*.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors acknowledge the support given by the Natural Products Laboratory and Molecular Biology Laboratory, Department of Biochemistry of the Universidade Federal of Pernambuco (UFPE), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thanks to Instituto Chico Mendes de Conservação da Biodiversidade/Brazilian Institute for Biodiversity Conservation (ICMBio) for collecting permits (Nº 26743-2; 26745-2).

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