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Cytotoxic and antioxidant capacity of extracts from *Vaccinium meridionale* Swartz (Ericaceae) in transformed leukemic cell lines

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ABSTRACT

Leukemia is one of the most common malignancies in children and represents a challenge to increase life expectancy. Many studies have been conducted with the aim to find natural compounds to fight cancer; however, chemotherapy is still the most widely used treatment despite side effects as harmful to patients. In this paper the antioxidant and cytotoxic capacity of Agraz extracts (*Vaccinium meridionale*) in OCI AML3 and MOLT4 cells using parallel Doxorubicin was evaluated. Extractions were made using 95% methanol, methanol-distilled water (1:1), distilled water and juice. Antioxidant capacity was performed by radical cation 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid and 2,2-diphenyl-1-picryl-hydrazyl-hydrate methods, and phenols content was determined by Folin-Ciocalteu. The cytotoxic effect was evaluated on the viability of OCI AML3 and MOLT4 cells using 96-well plates for cell seeding by hemocytometer to 96h. The antioxidant capacity expressed as 50% inhibitory concentration was 1.7 ± 0.1 and 4.5 ± 0.4 mg/L for methanol extracts by radical 2,2-diphenyl-1-picryl-hydrazyl-hydrate and radical cation 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, respectively. The phenol content was found on 104.7 ± 4.0 and 1669.7 ± 82.6 mg AG/100g for the methanol extract and lyophilized juice, respectively. A decrease in viability of OCI AML3 (24.4%) and MOLT4 (23.0%) cells was found when using Agraz methanolic extract, with Doxorubicin was 98.8 % and 85.0% respectively.

INTRODUCTION

Antioxidant compounds show a great ability to scavenge free radicals contributing to the prevention of cardiovascular and neurological diseases among many others chronic diseases including cancer (Sutachan *et al*., 2012). Chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases are characterized by an enhanced state of oxidative stress, which may result from the overproduction of reactive species and/or a decrease in antioxidant defenses.

The search for new chemical entities with antioxidant profile is still thus an emerging field on ongoing interest. The high diversity of plant species in Colombia has proven to be a source of many organic molecules with pharmacological properties (Thomas *et al*., 2012). Studies in some fruits and vegetables have shown to have a high content of antioxidants which has been recommended for frequent consumption. The current trend is the use of natural antioxidants so they can be used in food industry since it is presumed they do not cause health problems (Garzón *et al*., 2010; Abreu *et al*., 2014). Group of molecules commonly found in studies phytochemicals are flavonoids, these are characterized by their ability to scavenge free radicals which cause oxidative stress (Gaviria-Montoya *et al*., 2009) attributing them the prevention of cardiovascular, circulatory, cancer and neurological diseases among others (Alvarez-Castro *et al*., 2003; Sequeda-Castañeda *et al*., 2016). In the last two decades, studies on the antioxidant and cytotoxic capacity of plants and fruits have considerably increased due not only to the need to improve human health but also due to the development of new techniques for chemical analysis.

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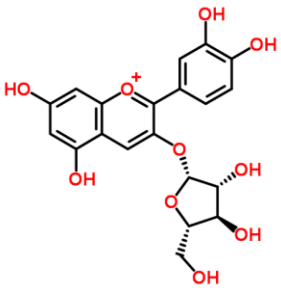

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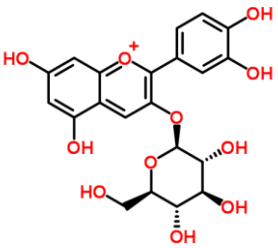

Cyanidin 3-glucoside
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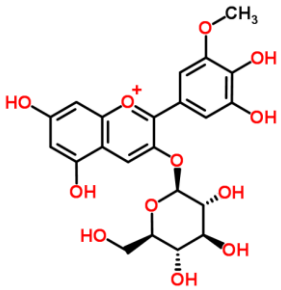

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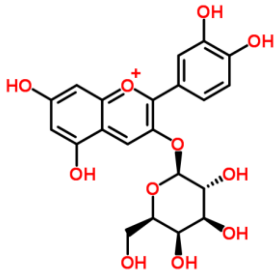
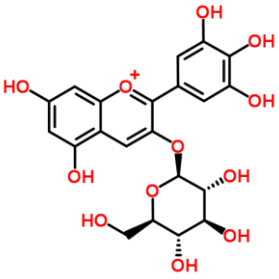


Cyanidin 3-galactoside (Ideain)
ACD/IUPAC Name: 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-chromeniumyl β-D-galactopyranoside



Delphinidin 3-O-glucoside (Myrtillin)
ACD/IUPAC Name: 5,7-Dihydroxy-2-(3,4,5-trihydroxyphenyl)-3-chromeniumyl β-D-glucopyranoside

Fig. 1: Compounds from *Vaccinium meridionale* Swartz (Ericaceae).

Table 1: Anthocyanin's present in *Vaccinium meridionale* Swartz (Eriaceae).

Metabolite	Quantity	Method	Reference
Cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, delphinidin 3-O-glucoside, petunidin 3-glucoside.	201 mg per 100 g fresh weight	UV-Vis	(Gaviria-Montoya et al., 2009)
	329 mg per 100 g fresh weight	HPLC-DAD. HPLC-ESI/MS-MS	(Garzón et al., 2010)
	151 mg per 100 g fresh weight	UV-Vis	(Maldonado-Celis et al., 2014)
	539 mg per 100 g dry weight	HPLC-UV	(Lopera et al., 2013)

Regarding to different species of blueberries, several researches have determined the antioxidant capacity in different species (Canter *et al*., 2004; Prior *et al*., 1998) specifically in *Vaccinium floribundum* Kunth. (Vasco *et al*., 2009) in wild blackberries (Deighton *et al*., 2000), unripe fruits (Garzón *et al*., 2010) and other different kinds of berries (Kähkönen *et al*., 2001). One of the main compounds found with cytotoxic effect in them are anthocyanin's (Figure 1, Table 1) (Garzón *et al*., 2010; Gaviria-Montoya *et al*., 2009; Kamei *et al*., 1995; Lopera *et al*., 2013; Maldonado-Celis *et al*., 2014; H. Wang *et al*., 1999). Agraz has in its phytochemical composition cyanidin 3-glucoside at levels up to 329 ± 28 mg/100 g (Garzón *et al*., 2010) compound to which it owes its high antioxidant potential of 45.5 ± 2.3 mol TE/g; in addition to this antioxidant effect. The *Vaccinium uliginosum* agraz has showed high cytotoxicity in colon cancer strains (Zu *et al*., 2010). Similarly, studies realized with cranberries have showed antioxidant-cytotoxic synergistic effect on ovarian cancer cells (Singh *et al*., 2009); using berries of French forests similar results have being achieved (Bendaoud *et al*., 2010), demonstrating the association between the antioxidant potential and cytotoxicity (Wang *et al*., 2007) due to reactive oxygen species are involved in common pathways such as AP-1 and NF-kB , showing proapoptotic, antiinflammatory and anti-proliferative effects (Circu *et al*., 2010; Yang *et al*., 2001). Some anthocyanins and their glicones such as cianidine, pelargonin, delfidin exhibit antiproliferative and proapoptotic activities on gastric HT-29 adenosarcoma and colon Caco-2 cancer (Yi *et al*., 2005). Even though many studies have been conducted to find natural methods to fight cancer, chemotherapy keeps being the most common treatment for cancer despite the side harmful effects. It is known that using Doxorubicin provides a diminution of cell response between 40 and 50%, but in turn causes nausea, mucositis and neutropenia risk of congestive cardiomyopathy (Winchester, 2001). In this research, we use Doxorubicin to compare and the aim was to evaluate the antioxidant capacity and potential cytotoxic effects of Agraz extracts on OCI AML3 and MOLT-4 cells and its possible application as an alternative treatment to prevent and / or fight cancer.

MATERIALS AND METHODS

Selection of plant material

Ripe fruits of the Colombian blueberry (*Vaccinium meridionale* Swartz) commonly Agraz were selected without mechanical or microbiological changes, with good color characteristics, fleshy and edible appearance (Obtain commercially in Department of Boyacá into Santa Bárbara Zone in Tinjacá region). The National Herbarium of Colombia has classified this species under the voucher COL208724.

Preparation of the Agraz Extract

100 grams of ripe fruits were steeped in 200 mL of MeOH (95%, acidulated with clorhidric acid 0.5 N to hydrolyze flavonoids glycosides), 200 mL MeOH : H_2O (1:1) and 200 mL of distilled H_2O for 48 hours. The extracts were filtered using a cotton cloth, whatman filter paper # 1 and concentrated by rotary evaporation (90 rpm and 45 °C) to syrup, which is subsequently lyophilized and stored at -80 °C. The juice obtained by crushing the fruit was filtered, one part was preserved and another lyophilized (Raaman, 2006; Thangaraj, 2016).

Preliminary phytochemical analysis

The methanol extract (568 mg), methanol-water extract (485 mg), aqueous extract (402 mg) and juice (333 mg) were dissolved in 5 ml of ethanol (EtOH) to perform the Lieberman-Burchard test for steroids and sterols, Salkowski to terpenes, Baljet to terpenes and sterols, ferric hydroxamate for sesquiterpenlactones, Shinoda and ferric chloride for flavonoids, anthrone to flavonoid glycosides and Dragendroff for alkaloids (Raaman, 2006; Thangaraj, 2016).

Total phenols

The determination of total phenols was performed using the colorimetric method of Folin-Ciocalteu proposed by Singleton and modified by Ortiz-Ardila (Singleton et al., 1999; Ortíz-Ardila *et al*. 2017). Two milligrams of each separately lyophilized extract were placed in 50 mL volumetric flasks, adding deionized water until dissolved. Then a 0.5 ml aliquot of each of these solutions were added 0.75 ml of Folin-Ciocalteu 1.0 N, allowed to stand at room temperature for 5 minutes and then added 0.75 ml of sodium carbonate 20%. The final solution was stored at room temperature in dark for 90 minutes. After this time the absorbance was measured at 760 nm using a spectrophotometer Thermo Genesys 10s. Results were expressed as milligrams of Gallic Acid per 100 grams of extract (mg GA/100 g).

Antioxidant activity

The antioxidant capacity was evaluated by the methods DPPH (1,1-Diphenyl-2-picryl-hydrazyl) and ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). 10 mg of the radical DPPH were diluted in 10 mL of MeOH and analytical absorbance at 516 nm was adjusted to 0.75 ± 0.05 by dilution (Brand-Williams *et al.*, 1995; Ortíz-Ardila *et al*., 2017). The lyophilized extract was diluted in deionized water to obtain a 5000 ppm stock and prepared 5 different concentrations: 50, 500, 1500, 3000 and 4500 ppm. From each solution, 25 µL were added to 975 µL of radical DPPH previously prepared to reach a final volume of 1 mL. The steady state was reached in 30 minutes, and the inhibition % was calculated as follows: % I = [(Ao-Ae)/Ao] x 100, where Ao corresponds to the absorbance of the solution of the radical without extract and Ae is referred as the absorbance to the radical plus the extract.

With the percentages of inhibition, scatterplots were obtained (% inhibition vs. concentration of the extract), in order to get the equation of the line (Y=mX+b) from which the 50% inhibitory concentration (IC_{50}) was calculated: (50-b)/m, being b the cut point in the Y axis, and m is the slope. The results were compared using Trolox and vitamin C (Kuskoski *et al*., 2005). For the Agraz juice (not lyophilized extract) the antioxidant capacity was determined by adding different volumes (10µL, 20µL, 30µL, etc). To calculate the values of the density and total solids in the juice AOAC 920.151 method and AOAC 950.28 method respectively were used (AOAC, 2011a, 2011b; Rodríguez-Rodríguez *et al*., 2012). From the value of the total solids and density of the juice the IC_{50} was found.

ABTS radical was prepared by dissolving 10 mg of the compound in 10 mL of water and then 2.4 mg of potassium persulfate were added. The prepared solution was left at room temperature 16 hours in dark until find an absorbance of 0.72 ± 0.02 at a wavelength of 735 nm (Re *et al*., 1999; Ortíz-Ardila *et al.*, 2017). Five different concentrations of each extract between 100 ppm and 4000 ppm from a 5000 ppm stock; the absorbance vs. time graphic was performed to determine the steady state. The inhibition % was calculated and the IC_{50} was obtained from the equation of line. For the Agraz juices the same procedure was carried out.

Measurement of the effect on OCI-AML3 and MOLT4 cells viability

Cell lines OCI-AML3 and MOLT4 were maintained in RPMI-1640 medium containing 5% FCS, 1% glutamine and 100 units/mL penicillin incubated at 37 °C and 5% CO_2. Characterization of the viability effect of the Agraz extracts and Doxorubicin was measured at the third day of culture. From the homogenized contents a 1000 µL aliquot of the cell suspension were plated and the cells were counted by hemocytometer (Phelan *et al*., 1997). Tests were performed in 96-well plates by seeding 100 µL of RPMI plus cells, adjusting its concentration to 10^6 cells/well (Handbook, 2011; Licor, 2010).

The lyophilized agraz methanol extract was applied to the wells at concentrations of 10, 50 and 100 µg/mL (using water as dissolvent) for both cell lines, as a control a concentration of 0 µg/mL was used. The results were correlated with viability test using Doxorubicin at concentrations of 10, 25 and 50 ng/mL, as a control concentration 0 ng/mL was used. After dosing, each plate was stored in an incubator at 37 °C with 5% CO_2 and the live cells

were counted by hemocytometer with trypan blue (Phelan *et al*., 1997; Thorkild *et al*., 1985) at 48, 72 and 96h. The viability percentage reduction caused by the extract and Doxorrubicin was calculated, results were expressed in µg/mL of extract and in ng/mL of Doxorubicin (Licor, 2010; Phelan *et al*., 1997).

Statistical analysis

The software IBM SPSS Statistics 19 was used. All experiments were done in triplicate (n=3) and results were expressed as means ± standard deviations. Analysis of variance was analysed using the Tukey HSD test based on significant difference of $p < 0.05$.

RESULTS AND DISCUSSION

Agraz extracts

The extracts of different polarities were obtained from 100 g of fresh fruit. The extract yield for each polarity was 2.3 ± 0.5 %, 1.8 ± 0.4 % and 1.2 ± 0.3 % for methanol extract, methanol-water extrac (1:1) and water extract, respectevely.

Preliminary phytochemical analysis

The results of qualitative chemical tests (Table 2) show high similarity between the compounds present in the Agraz juice and extracts using solvents such as methanol, methanol-water (1:1) and water. The compounds detected correspond to phenols and tannins, flavonoids, terpenes, terpene lactones, coumarins and steroids.

Table 2: Preliminary phytochemical analysis.

Test	Juice		Extract in		
	no liophylized	liophylized	MeOH	MeOH-H_2O*	H_2O
Phenols and tannins					
Gelatin - salt	+	+	-	-	+
Ferric chloride	+	+	+	+	+
Saponins					
Foam	-	-	-	-	-
Alkaloids					
Dragendorff	-	-	-	-	-
Flavonoids					
Shinoda	+	+	+	+	+
Cardiotonics					
Baljet	+	+	+	-	-
Triterpenes and steroids					
Salkowski	+	+	+	+	+
Lieberman - Burchard	+	+	+	+	+
Terpene lactones and coumarins					
Ferric hydroxymate	+	+	+	+	+

* MeOH-H_2O = Methanol-Water (1:1). (+): Presence. (-): Absence.

Antioxidant capacity and total phenols content

The results show the largest amount of total phenols for the not lyophilized and lyophilized Agraz juice 233.4 ± 7.6 and 1669.7 ± 82.6 mg GA/100g respectively, compared to the extracts obtained with solvents such as methanol (104.7 ± 4.0 mg GA/100g), methanol-water (32.9 ± 3.5 mg GA/100g) and water (79.4 ± 6.4 mg GA/100g) (Table 3).

Table 3: Total phenolic content in Agraz extracts and juice.

mg GA/100g *	Juice		Extract in		
	no liophylized	liophylized	MeOH	MeOH:H_2O [a]	H_2O
Mean	233.4	1669.7	104.7	32.9	79.4
StdDev	7.6	82.6	4.0	3.5	6.4
RSD. %	3.3	4.9	3.8	10.7	8.1

* mg Gallic Acid per 100 g of sample. 3 determinations average. [a] Methanol-Water (1:1)

Garzón *et al*., in 2010 reported 758.6 ± 62.3 mg GA/100g in methanolic extract after subjecting the fruits to liquid nitrogen; not lyophilized and lyophilized Agraz juice showed the highest concentration of total phenolic compounds compared to other berries such as *Vaccinium floribundum* of Ecuador whose amount is 882 mg GA/100g (Vasco *et al*., 2009), *Vaccinium myrtillus* of North America in 525 mg GA/100g and between 190-473 mg GA/100g for *Vaccinium corymbosum*, *Vaccinium asheiy* and *Vaccinium angustifolium* (Garzón *et al*., 2010). In *Vaccinium meridionale* 86 ± 4 mg GA/100g in the methanol extract was reported (Sequeda-Castañeda *et al*., 2016), whereas in this study for the same type extract was 104.7 mg GA/100g. Among the extracts, the methanolic has the best IC_{50} and has the highest total phenolic content. The amount of total phenols in the different extracts was lower than that found in the juice (without lyophylized and lyophilized). Possibly due to differences in solubility to total phenols present in the solvents used. Additionally, the lyophilization process substantially increase the phenol content in the lyophilized juice because the process removes the water present in the juice and increase the content of solutes (solids). All samples have high antioxidant capacity. The extract with the highest antioxidant capacity expressed as IC_{50} correspond to the methanol extract with values of 1.7 ± 0.1 mg/L (DPPH method) and 4.1 ± 0.3 mg/L (ABTS method). While controls showed values of 3.1 ± 0.2 mg/L (ABTS method) and 20.8 ± 1.2 mg/L (DPPH method) for Trolox and 2.4 ± 0.2 and 3.5 ± 0.3 mg/L for ABTS and DPPH methods, respectively for ascorbic acid (Table 4). In another study the IC50's were 3.8 ± 0.3 and 22.9 ± 5.4 mg/L in ABTS and DPPH methods, respectively, for methanol extract (Sequeda-Castañeda *et al*., 2016); these values are lower than those reported in this study. The high antioxidant capacity is probably to its higher content of anthocyanins, phenols and a different polyphenols (Lopera *et al*., 2013).

Table 4: Antioxidant capacity for Agraz juice and extracts*

IC_{50} [a] (mg/L)	Agraz extracts in:						Trolox [c]	
	MeOH		MeOH:H_2O [b]		H_2O			
	ABTS	DPPH	ABTS	DPPH	ABTS	DPPH	ABTS	DPPH
Mean	**4.1**	**1.7**	74.5	16.9	17.6	3.9	3.1	20.8
StdDev	0.3	0.1	4.6	1.1	1.3	0.3	0.2	1.2
RSD. %	7.3	5.9	6.2	6.5	7.4	7.7	6.5	5.8

IC_{50} [a] (mg/L)	Juice				Vitamine C [b]	
	no liophylized		liophylized			
	ABTS	DPPH	ABTS	DPPH	ABTS	DPPH
Mean	4.5	5.5	23.7	35.6	2.4	3.5
StdDev	0.4	0.5	1.3	2.7	0.2	0.3
RSD. %	8.9	9.1	5.5	7.6	8.3	8.6

* 3 determinations average. In bold number the best antioxidant capacity. [a] Inhibitory concentration 50%. [b] Methanol-Water (1:1). [c] Controls used in antioxidant capacity

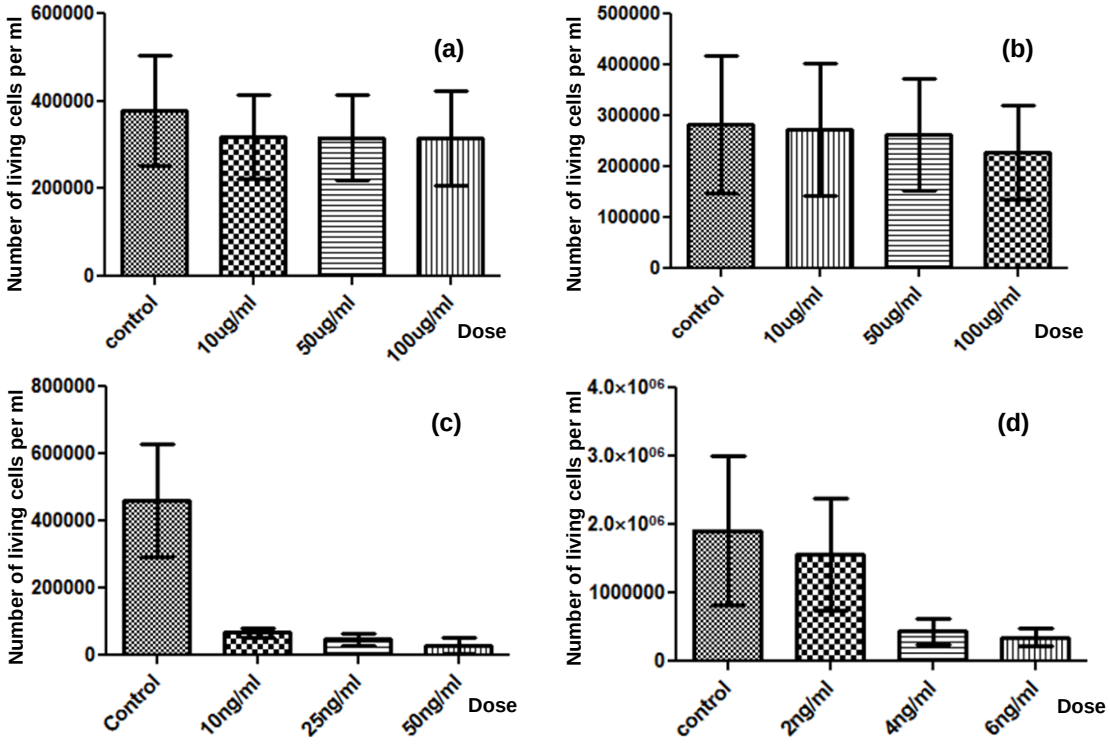


Fig. 2: Cell viability of (a) OCI AML3 cells with methanolic extract of Agraz. (b) MOLT4 cells with methanolic extract of Agraz. (c) OCI AML3 cells with Doxorubicin. (d) MOLT4 cells with Doxorubicin. Three different experiment with three replicates (wells) each one (n=3). Significance values ($p>0.05$) was calculated with Tukey's test.

Effect of the Agraz extract in MeOH and Doxorubicin on cell viability of OCI-AML3 and MOLT4 cells

The methanol extract was selected to evaluate the effect on cell viability because it presented the best antioxidant capacity (IC_{50} of 4.1 and 1.7 mg per L for ABTS and DPPH methods, respectively). The viability in OCI-AML3 cells decreased by 11.8, 20.8 and 24.4% for doses of 10, 50 and 100 g/mL respectively. While the MOLT4 cells showed 2.4, 12.8 and 23.0% with doses of 10, 50 and 100 g/mL. The effect of Doxorubicin in inhibiting the viability of OCI-AML3 cells was 84.1, 91.3 and 98.8% for doses of 10, 25 and 50 ng/ml respectively. In MOLT4 cells the decrease was 29.0, 81.0 and 85.0% when using doses of 2.0, 4.0 and 6.0 ng/mL, respectively (Figure 2). The decrease OCI-AML3 cells viability with the methanolic Agraz extract was statistically significant (sig 0.000 $p\leq0.05$) among all the means of the tested concentrations. While the decrease for MOLT4 cells was not significant (sig 0.969 $p\leq0.05$). Observed decrease in cell viability percentage was much lower compare to standard drug doxorubicin in all cases. The mechanism by which the extract inhibits cell proliferation may be due to the induction of apoptosis (Shih *et al*., 2005) by NF-kB and AP1 pathways (Wang *et al*., 2007) or by inhibition of certain enzymes such as cyclooxygenase (Seeram *et al*., 2003), however further investigation must be addressed.

Antioxidants used in leukemia and their relation with chemotherapy side effects

Some plants and fruits rich in antioxidant content as ginger root, grapes, carrot, black and blueberries etc. have been

used for leukemia treatment (Saedi *et al.*, 2014). Different authors reported that antioxidant use during chemotherapy decreases mucositis (Wadleigh *et al*., 1992) and that a lower consumption of vitamins and antioxidants was related to an increased risk of a delay in therapy or of experiencing hematologic or nonhematologic toxicity in kids with leukemia (Kennedy *et al*., 2004).

However, remains to clarify the basis of the mechanisms by which antioxidants would be mediating the reduction of some of the side effects of chemotherapy in patients with leukemia. Thus, our study provides a basis for continuing research on the potential for dietary and pharmaceutical antioxidants to enhance the effects of chemotherapy and decrease toxicity without interfering with oncological proceeding.

CONCLUSION

The Agraz juice and methanolic extract show a high antioxidant value per the results of the ABTS and DPPH assays. Even though the cytotoxic tests with the Agraz methanolic extract did not yield high percentages of decreasing viability of OCI-AML3 and MOLT4 cells, the Agraz juice -both lyophilized and non-lyophilized- might be a potentially cytotoxic source because of its high antioxidant capacity and total phenolic content. Regarding to Doxorubicin, this showed a greater effect in decreasing the proliferation of OCI-AML3 (98.8%) and MOLT4 (85%) cells than that obtained with the Agraz methanolic extract for OCI AML3 cells (24.4%) and MOLT4 (23.0%).

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Conflict of Interests: There are no conflicts of interest.

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