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Antioxidant activity and cytotoxicity of *Vacciniun corymbosum L.* cultivated in Tucumán, Argentina

María Paola Muratore Lorenzi [1], Cesar Emmanuel Ale [1, 2] and María Alejandra Moyano [1, *]

[1] *Faculty of Biochemistry, Chemistry and Pharmacy, Chair of Quality Assurance of Drugs and Medications, National University of Tucumán, Tucumán, Argentina.*
[2] *Faculty of Biochemistry, Chemistry and Pharmacy, Chair of Microbiology, Faculty of Agronomy and Zootechnics, Chair of Organic Chemistry, National University of Tucumán, Tucumán, Argentina.*

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Abstract

Among berries, blueberries (*Vaccinium corymbosum L.)* are considered one of the main sources of phenolic compounds and are appreciated for their high antioxidant activity. Due to the apparent relationship between phytochemicals in plants to the prevention of chronic diseases, the study of the content and physiological activity of phenolic compounds and other bioactive metabolites present in blueberries is interesting.

In this work, Tucuman blueberries of the O'Neal variety were used, out of which four different extracts were obtained. One of them was obtained by sequential leaching with hydroalcoholic mixtures from frozen fresh fruits (E1), a second extract from aqueous leaching (E2), and acetone as solvent (E3). Spectrophotometric techniques were performed to quantify Total Phenols, Total Flavonoids and Anthocyanins. The extracts presented a similar quantitative profile of phenolic compounds. The antioxidant activity of the extracts obtained was evaluated using two different techniques (ABTS•+ and DPPH•). The extracts showed a free radical inhibitory activity and a similar reducing power.

Through the acute toxicity test (24h) of the organic and aqueous extracts from the blueberry on *C. elegans*, .it was possible to determine that the extracts under study do not show toxicity for further study.

Keywords: Total phenols; Anthocyanins; Antioxidant capacity; Cytotoxicity; *Vaccinium corymbosum L*

1. Introduction

The search and use of natural bioactive compounds, such as antioxidants anti-inflammatory or antimicrobial, has gained great interest in the last years as a consequence of the pandemic of Covid-19 [1, 2, 3]. In the case of the antioxidants, recent studies have shown its use as adjuvant for the prevention and treatment of COVID-19 [4, 5, 6]. In addition, clinical trials confirm that the activity of numerous natural antioxidants is higher than the one from pharmacological supplements which contain synthetic antioxidants [7, 8, 9, 10, 11].

On the other hand, antibiotics have been excessively prescribed on patients hospitalized with COVID-19, which may contribute to a higher resistance of the pathogenic microorganisms [12], a current issue which is approached from various angles [13, 14]. To address this, JPIAMR (Joint Programming Initiative on Antimicrobial Resistance) promotes the research on the antimicrobial resistance, with a current focus on the integration of new options (products, strategies

* Corresponding author: María Alejandra Moyano
Faculty of Biochemistry, Chemistry and Pharmacy, Chair of Quality Assurance of Drugs and Medications, National University of Tucuman, Tucuman, Argentina.

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and methods). For this purpose, plants contribute in a significant way, as promising sources of bioactive substances with a wide range of biological properties [15].

Blueberries are well-known for their phytotherapeutic attributes, used for medicinal purposes for ages and are currently interesting research models to highlight natural attributes which are beneficial for the health [16].

In Argentina, blueberry production is relatively new and most of it is exported as fresh fruit to the Northern hemisphere. However, due to weather conditions (late frost damage, rain during harvest) or due to logistics and commercialization problems, all the potential production cannot be harvested or commercialized, this fruit could be used in the pharmaceutical, dermaesthetics or food industry, using its biological attributes for the creation of natural products with a high market value [17].

This trend offers a remarkable position to the concentrated vegetable extracts in active substance which offer these benefits. For this reason, ANMAT (Resolución (MSyAS) N° 144/98; Disposiciones ANMAT Numbers: 2673/99; 2819/04-Anexo VIII) incorporates and defines Plant Based Drugs as products obtained from them (tinctures, extracts, digested, pulverized or others) in which procedures such as extraction, distillation, purification, drying, etc. Therefore, each preparation is entirely considered as an active substance.

The extracts obtained from blueberries represent a valuable source of secondary metabolites as phenolic compounds, with various attributes, among which the antimicrobial and antioxidant activity is highlighted [18, 19], allelopathic y anti-inflammatory [20]. Different factors affect the secondary metabolites synthesis in the plants such as stress, flowering stages, plant age and type of soil [21]. For this reason, this work has the intention to quantify the present secondary metabolites (total phenols, flavonoids and anthocyanins) and determine the antioxidant capacity in extracts from (*Vaccinium corymbosum L.)* (Cultivar O' Neal). In addition, evaluate their safety to be prescribed as phytotherapeutic in *in vivo.* trials.

2. Material and methods

2.1. Vegetable material

The fruit picking (*Vaccinium corymbosum L.)* (Cultivar O' Neal) was performed in October, 2018 in El Corralito, Localidad de Juan Bautista Alberdi, Provincia de Tucumán (geo localization -27°33'74.96"S, -65°69'28.16"W). The species under study was characterized by INTA (Instituto Nacional de Tecnología Agropecuaria).

Immediately after picking the fruits, they were placed on polyethylene terephthalate trays to reduce the damage post-harvest. Later they are stored at 3 ± 0.5 º C until transported to the laboratory. They were placed in a cooling chamber (0 ± 0.5 º C) until its use in the different planned experiences. The lapse of time between the fruit picking and the experiences was no longer than a week to preserve its characteristics.

2.2. Extract preparation

The extracts were obtained through sequential extraction with solvents with increasing polarity by leaching process. The technique was performed with some changes according to the instructions from Soberón *et al*., 2010 [22]. The fruits were washed in sieves with bidistilled water and were drained with soft shaking.

2.2.1. Hydroalcoholic extract

An IVA of 40 x 50 mm glass column, with PTFE (polytetrafluoroethylene) robinette, was packed with 150 g shredded material respectively. The shredded material was moistened with absolute ethanol p. a. (MERK), and was kept for 24 h away from light. Once the shredded material was moistened, the leaching was performed, collecting the leaching in an Erlenmeyer (1st leaching). Later, the leaching with different decreasing alcoholic content was performed: ethanol 70° and 45° collecting the leaching on the former leaching (E1). The last leaching was concentrated in rotatory evaporator using an equipment Büchi R-210, at 40 °C and stored at -20 °C. The aqueous part of the extract was eliminated by lyophilization (Freezone 6, Labconco, USA). In the end, the dry residue was weighed and stored in an amber colored jar at -20 °C until its later use. It was called extract E1.

2.2.2. Aqueous extract

Once the fruits were washed they were milled with a mechanical mill, after that they were in maceration with bidistilled water for 24 h, later a double filtration was performed on filter paper and it was frozen at -20°C to be lyophilized,

following a modified protocol from the work of Kraujalyte *et al*, 2015 [23]. The resulting lyophilized material (E2) was weighed in an analytical scale, later sealed and stored at -20°C until its use.

2.2.3. Acetonic extract

The solvent used was acetone p.a. (Merck). The extracts were formulated with a combination of 150 g of shredded fruit in 100 ml of solvent (acetone: water; 70: 30). The shredded material was moistened with acetone and protected from light for 24 h and later the leaching was recovered and the solvent eliminated through rotatory evaporator, until dryness at 40 °C. Later, it was frozen at -20°C to be lyophilized (E3), weighed and stored in the same conditions before described.

2.3. Extraction yield

The extraction yield (EY) was calculated considering the amount of dry extract obtained (P_{ES}), after the lyophilization, in relation to the amount of shredded fruit initially (P_{MP}) expressed in percentage**:**

$$RE (\%) = (P_{ES} / P_{MP}) * 100$$

2.3.1. Preliminary detection of phenolic compounds in the extracts.

The qualitative detection of phenolic compounds in the extracts was carried out through a test with a solution of $FeCl_3$ 5 % (grade analytical, Sigma-Aldrich). This reaction allows to recognize the presence of phenolic compounds in the vegetable extract, both in aqueous or ethanolic solutions [24]. To cause this reaction at a fraction of 1 ml of extract (dissolved in ethanol or water) 3 drops of $FeCl_3$ were added.

Total phenolic compounds analysis

The antioxidant activity is related to metabolites which contain phenolic groups, they were determined through an oxide colorimetric reaction -reduction according to García *et al*., 2011 [25] and further modifications. Stock Solutions (1 mg/ml) were prepared of each of the extracts, they were mixed with distilled water to obtain a final volume of 2 ml and 0.2 ml of the Folin-Ciocalteu reagent (analytical grade, Merck) was added. 2 minutes later, 0.8 ml of aqueous solution Na_2CO_3 2% (p/v) was added. The samples were incubated at 25 °C for 10 minutes next, the absorbance readings were performed on 760 nm in a UV-Visible spectrophotometer (Carry 60 UV-Vis, Agilent Technologies), using gallic acid (analytical grade, Merck) as standard (optimal range: 10 - 90 µg/ml). The total phenols content was determined through the regression equation $y = 0.0012 x + 0.0221$ (R^2 of *0.9998*). The results expressed as mg Equivalents of Gallic Acid (EAG) by 100 grams of dry extract (mg EAG/100 g d.e.).

2.3.2. Determination of the total flavonoids content (TFC)

The technique proposed by Arvouet-Grand y col. was used, 1994 [26] and further modifications: 1.5 ml of Stock Solution (1 mg/ml) was mixed with the same amount of a methanolic solution of $Al Cl_3$ 2% (p/v) (analytical grade, Merck). The mixture was for 10 minutes at room temperature. After this time, the absorbance was measured at 415 nm in UV-Visible spectrophotometer,

The TFC was estimated by comparison with the optical density values obtained with different concentrations of quercetin used as standard (5 - 40 µg/ml). (analytical grade Sigma-Aldrich) through the equation: and = 0.0235 x + 0.0319; ($R^2 = 0.9986$). The results were expressed in mg Quercetin Equivalent (QE**)** per 100 grams of dry extract (mg QE/100 g d.e.).

2.3.3. Determination of the anthocyanins content

The quantification was carried out by the differential method of pH [27] with some modifications. Interpretations in UV in the range of 400 - 700 nm where values ≤ 0.8 UA were considered. 300 µl extract stock solution was taken and 600 µl of buffer pH 1 (0.025 M de KCl) were added on the other hand, 300 µl of stock solution was taken and 600 µl of buffer pH 4.5 (0.4 M Sodium acetate) was added. Interpretations in UV in the range of 400 - 700 nm were made for samples with both ph. Interpretations at pH 1 and at pH 4.5 at maximum absorbance wavelength at pH 1, which correlated at 520 nm, as well as interpretations at 700 nm [28]. The results were expressed as mg Cyanidin Equivalents 3-glycoside (EC-3G) by 100 grams of dry extract (mg EMv-3OG / 100 g d.e.).

For the extraction of the anthocyanins concentration the pH differential formula was used:

$$A = (A_{\lambda 520nm} - A_{\lambda 700nm})_{pH\,1} - (A_{\lambda 520nm} - A_{\lambda 700nm})_{pH\,4.5}$$

Where;
$A_{\lambda 520nm}$, is the interpretation of the highest peak at pH 1 and pH 4.5.
$A_{\lambda 700nm}$, is the interpretation at 700 nm, both to pH 1 and pH 4.5.

To calculate the concentration in the original sample the following formula was used:

$$\text{Monomeric anthocyanin (mg/l)} = (A \times PM \times FD \times 1000)/(\varepsilon \times 1)$$

Where;
A: absorbance of the sample previously calculated.
DF: dilution factor (Final Volume/ Sample Volume).
MW: molecular weight of the Cyanidin-3-glycoside (449.2 g/mol).
Ɛ: molar extinction coefficient of Cyanidin-3-glycoside (26900).

2.4. Antioxidant Activity Assay

2.4.1. Inhibitory activity of the radical 2.2-azino-bis-(3-ethylbenzothiazoline)-6- ammonium sulphate (ABTS•+)

The radical $ABTS^{\bullet +}$ (analytical grade, Sigma-Aldrich) was generated using a reactive mixture with ABTS (3.5 mM) and potassium persulfate (1.25 mM) (analytical grade, Merck) in distilled water. The reactive mixture was prepared 12 h. before use and was protected from light. With the extracts, aqueous solutions were prepared at a concentration of 1 mg/ml. The absorbance of the solution of $ABTS^{\bullet +}$ was adjusted at 0.7 units (± 0.05) at 732 nm with buffer PBS (pH 7.3). 0.5 ml of each extract was placed in each tube, distilled water was added until reaching a volume of 1 ml, finally 0.9 ml of the solution of $ABTS^{\bullet +}$ was added; thus, reaching a final volume of 1.9 ml. As referent antioxidant agent a solution of ascorbic acid was used. The tubes were protected from light for 30 minutes. After this time, the reduction of absorbance at 732 nm was measured in spectrophotometer in relation to a control which does not contain vegetable extract. The results were expressed as extract concentration which produces 50 % of the radical purification (CI_{50}). [29, 30].

2.4.2. Inhibitory activity of the radical 1.1-diphenyl-2-picryl-hydrazyl (DPPH●)

1 mg/ml of each sample was weighed to determine the antioxidant activity of each extract (Stock Solution). On the other hand, 1.23 mg of DPPH• were weighed in a previously tared volumetric flask and 25 mL of DMSO was dissolved to obtain 125 µM, solution which was placed in a sonicator to be shaked protected from light.

25 µl of extract and 1 ml of radical 2.2-diphenyl-1-picrylhydracil (DPPH• 125 µM) were added in each tube. An ascorbic acid solution was used as control. The tubes are kept covered and in the darkness, at room temperature for 30 minutes. After that time, in a UV-Visible Metrolab spectrophotometer was read 1700 at 545 nm. In every case, the determinations were carried out in triplicate and the antioxidant (% AA) of the DPPH•, was calculated following the formula:

$$\text{\%Antioxidant Activity (\%AA)} = 100 \times (Abs_{control} - Abs_{sample} / Abs_{control})$$

After discarding the samples with a percentage of antioxidant activity minor to 25 %, the graphs were performed % AA in relation to the starting concentration (1 mg/ml) and by the linear regression equation the value of IC_{50} was determined [25, 31].

2.5. Citotoxic assay

For the citotoxic assay an *in vivo* model with *Caenorhabditis elegans.* was used. This organism is a free-living nematode easy to grow in the laboratory and it has a short life-cycle of 3 days at 20 °C. The genome of *C. elegans* is almost completely sequenced; it is the only organism with a nervous system and a cell lineage completely mapped, it is a simple animal with a centralized nervous system of only 302 neurons, which makes its behavior a simple one. [32, 33].

The trial of lethal dose is a standard toxicological trial which allows to determine the concentration at which 50% of animals die. (LC_{50}).

The acute toxicity (24 h) of blueberries extract on *C. elegans* was evaluated. The three extracts, soluble in water (E1, E2, E3) were studied at four concentrations: 1000, 500, 250, 125 µg/ml. 6 cups were used for each concentration with 20 individuals each (total average of 120 in stage L4), 8 cups control (average 160). The Methodology from Bischof *et al.*, 2006 [34] modified.

After 24 h of exposure to the extracts, the worms were collected washing the plaque with 5 ml of water and they were transferred to a conic tube with a Pasteur pipette of sterile glass. Later, photos were taken to count them. The worm viability was determined on its movement: A worm that visibly moves is marked as alive. The worms that do not move and do not react to various taps are marked as dead.

The data is presented as percentage of alive worms against the extract concentration to determine the concentration CL_{50}.

2.6. Statistics Assay

An analysis of variance (ANOVA procedure) for the analysis of secondary metabolites and antioxidant activity was performed of a factor and, later, on the variables that presented a significant, difference; the Tukey's range test ($p < 0.05$) was used for multiple comparisons, verifying possible significant differences among the physicochemical variables.

For the citotoxic assay, the concentration data [Log (X)] were transformed for the EC50 calculations (non linear regression) using the GraphPad Prism 5.0 software.

3. Results and discussion

3.1. Extraction yield

The extraction yield is presented in graph 1. Solubility tests were performed on every extract, proving that all of them are highly soluble in water. Extracts E1 y E2 do not present significant differences between them as regards the extraction yield of 36.86 y 39.15% respectively. Only extract E3 presented a significantly inferior extraction yield than the rest (27.63%).

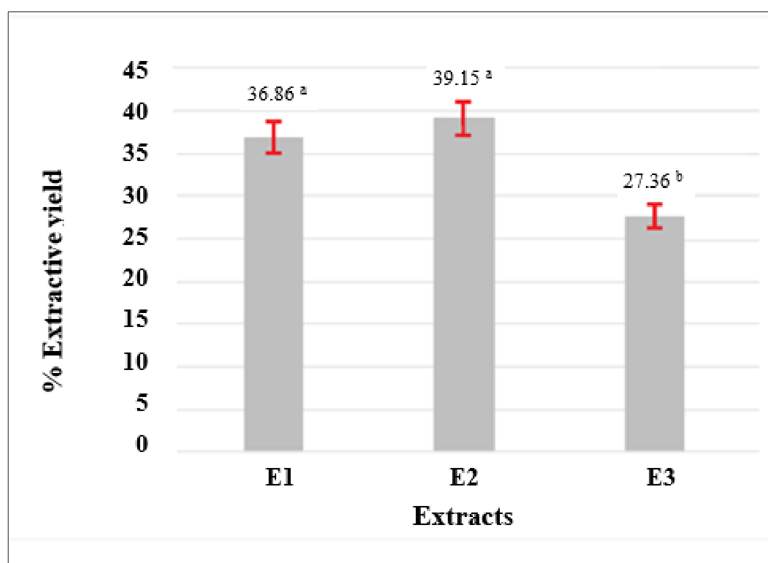


Figure 1 Extractive performance from blueberry extracts

These results would indicate a high recovery in the processed fruits which coincides with the report by Gozzi, 2011 [35] in blueberry foliar extracts *Vaccinium ashei* and in berries of the genus *Vismia* (*Guttiferae*) reported by Alvarez, 2008 [36]. The extraction of organic compounds in plants is directly related to the compatibility of these compounds with the solvent, in this way, when its polarity characteristics match the polarity of the solvent are extracted more easily. This is reflected in the extract yield results under study since the higher polarity of the solvent, the greater is the yield. On the other hand, a study carried out by Zapata, 2014 [37] says that the process variables such as pH, temperature, extraction time, type of solvent and proportion of raw material / solvent, have a significant influence in the extraction solid liquid, and therefore in the quantification of its secondary metabolites.

3.2. Quantification of Phenolic Compounds

The extracts were characterized considering its Total Phenols Content (TPC), Total Flavonoids (TF), Total Anthocyanins (TA). The comparison of the content of the different components is presented in Table 1.

Table 1 Quantification of different phenolic components present in blueberry extracts

Phenolic Constituents	**E1**	**E2**	**E3**
Total Phenolic Compounds (mg EAG/100 g e.s.)	3474.84 ± 1.20[a]	4027.34 ± 0.13[b]	2339.53 ± 0.56[c]
Total Flavonoids (mg EQ/100 g e.s.)	572.82 ± 0.23[a]	811.86 ± 0.16[b]	15.21 ± 1.97[c]
Total Anthocyanins (mg EC-3G/100 g e.s.)	685.49 ± 0.44 [a]	293.25 ± 0.70[b]	71.55 ± 2.03[c]

References: (mg EAG/100 g d.e.): equivalent milligrams of gallic acid per 100 grams of dry extract; (mg EQ/100 g d.e.): equivalent milligrams of quercetin per 100 grams of dry extract; (mg EMv-3OG / 100g d.e.): equivalent milligrams of Cianidin 3-glucuside per 100 grams of dry extract.

The greater content of total phenols extracted correspond to the aqueous extract E2 with 4027.34 ± 0.13 mg AG / 100 g d.e., followed by E1 with 3474.84 ± 1.20 mg AG / 100 g d.e. and E3 2339.53 ± 0.56 mg AG / 100 g d.e. The variance assay indicated that there exist significant differences in the concentration of total phenols in the studied extracts. In this way, E1 – E2, E1 – E3 y E2 – E3 differ significantly ($p<0.05$).

The Flavonoid content show that it is greater depending on the polarity of the solvent. The presence of flavonoids in each of the extracts was confirmed, being greater in extract E2 followed by E1 and very low in the extract E3 which is the acetonic (572.82 ± 0.23; 811.86 ± 0.16; 15.21 ± 1.97 mg QE / 100 g d e. respectively). The variance assay indicated that there exist significant differences in the concentration of total phenols in the studied extracts (Tukey: E1 – E2, E1 – E3 and E2 – E3 differ significantly, $p<0.05$).

The determination of anthocyanins shows that the el extract E1 presents content significantly greater 685.49 ± 0.44 than E2 y E3, 293.25 ± 0.70; 71.55 ± 2.03 respectively ($p<0.05$). These results are similar to those reported by Lillo, 2016 [38] for berries native of the South Cone of America specially for maqui (*Aristotelia chilensis*), and were greater to the concentrations reported in *V. corymbosum* of this region.

The quantity of phenolic compounds present in the extracts in the study is less than the quantity reported by Gozzi, 2011 [35] in Blueberries dry leaves *Vaccinium ashei* and berries of the genus Vismia, ~~[36]~~ Álvarez, 2008 [36]. When comparing the tested extracts to other of the same species form different regions they showed similar quantities of phenolic compounds in the hydroalcoholic extractions reported by Pesantes Arriola, 2021 [39] and greater than Lillo, 2016 [38]. The measurement of total phenols was most widely used methodology for a quick quantification in different types of vegetable extracts showing a high similarity with other measurements of flavonoids, mainly flavones, total flavonols and total anthocyanins and it is directly related to the antioxidant activity of the extracts of berries. The three extracts in the study present a wide difference in the content of different types of polyphenols specially in total anthocyanins which are the most important flavonoids in this type of fruits and give the berries a pigmentation between blue and red and other organoleptic characteristics.

3.3. Antioxidant Activity

The antioxidant activity was assessed with two different methods ABTS and DPPH (Table 2).

Table 2 Antioxidant capacity of blueberry extracts by the ABTS•+ radical and DPPH• radical decolorization methods

Extracts	**ABTS • +** **CI50 (µg/ml**	**DPPH** **% Inhibitión of DPPH•**
E1	21.57 ± 1.37 [b]	30.86 ± 1.23 [b]
E2	54.21 ± 1.41 [c]	115.29 ± 0.26 [c]
E3	89.54 ± 0.61 [d]	145.83 ± 0.41 [d]
Ascorbic acid	1.67 ± 1.58 [a]	1.55 ± 0.61 [a]

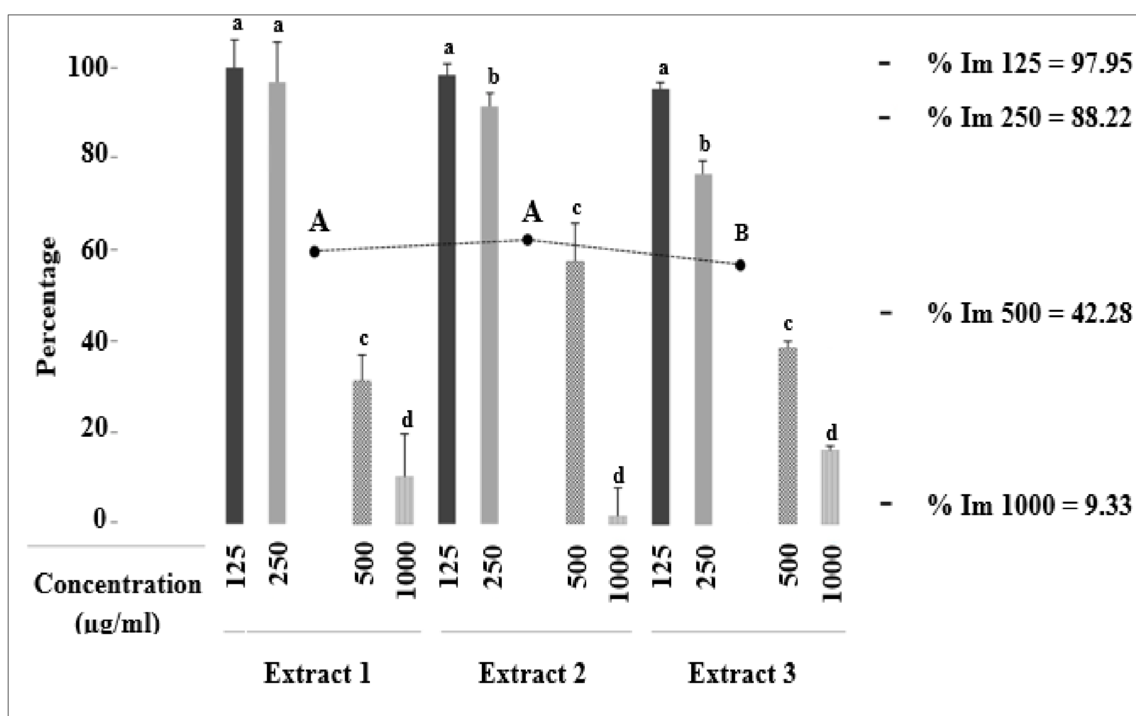
References: IC50 concentration of the extract that produces 50% radical clearance.

The antioxidant activity, measured with two different methods, was higher in aqueous extract E1 followed by E2 and then with remarkable inferior activity E3. There is no direct correlation between the phenolic content and the antioxidant activity in E1 and E2, the presence of other substances which are not determined in this study could contribute with the mentioned phenomenon, [40]. In every case analyzed a significant difference may be observed among the analyzed extracts (p-< 0.05). Values from ABTS CI50 (µg / ml) were to E1=21.57 ± 1.37; E2=54.21 ± 1.41; E3=89.54 ± 0.61. To DPPH E1= 30.86 ± 1.23; E2=115.29 ± 0.26 E3=145.83 ± 0.41.

As regards, the antioxidant activity, the berries native of the Southern Cone of America studied by Lillo, 2016 [38] present higher activity although the phenolic contents were similar, which could be associated to the fact that during the treatment, phenolic compounds of high polar were extracted together with sugars, inorganic salts and other non-active compounds to capture radicals.

3.4. Citotoxicity

In graph 2 the average percentage of live individuals after each treatment for the extracts E1, E2 y E3.



[a-d] different letters indicates significant differences between media percentage of alive *C. elegans* individuals for each concentration of extracts applied; [A-B] different letters indicates significant differences between media percentage of alive *C. elegans* individuals for each extract applied; %Im= media percentage of alive individuals under each treatment applied.

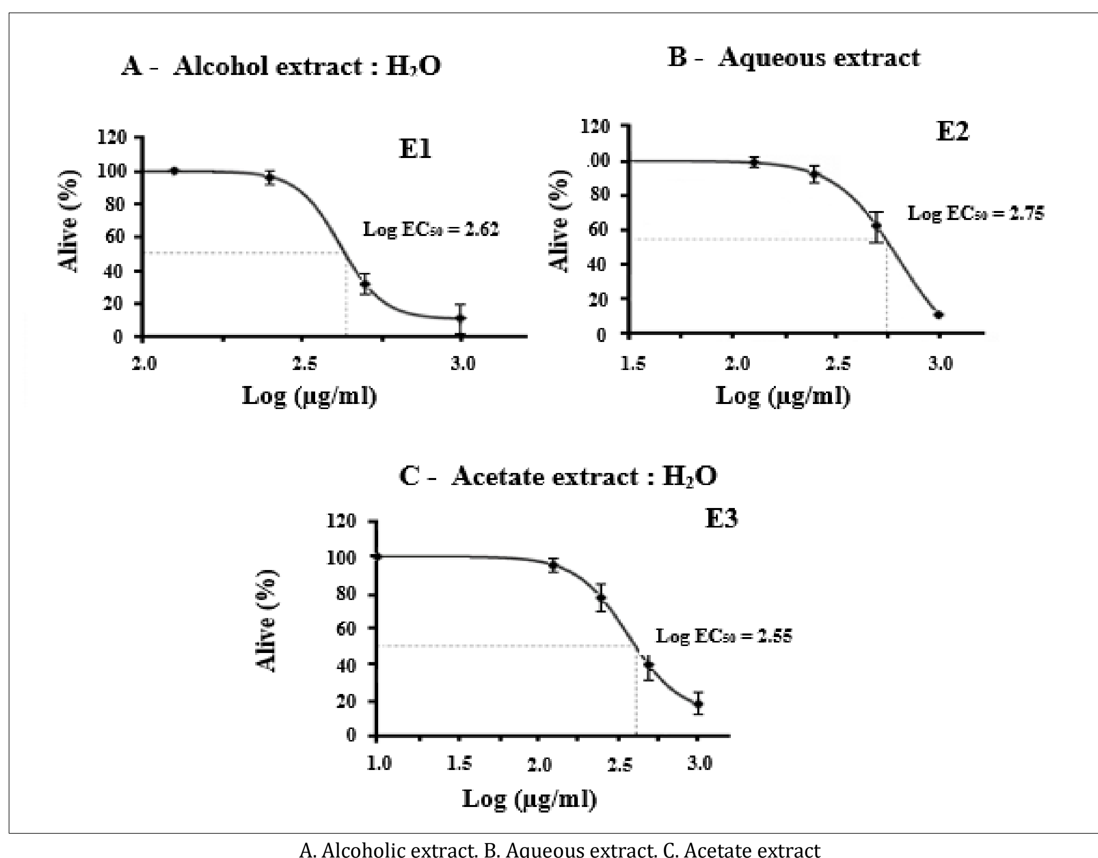
Figure 2 Percentage of individual alive (*C. Elegans*) versus blueberry extracts

In general, percentage of surviving individuals diminished when concentration was increasing, mainly in 1000 µg/ml in wich 9.33% of survival followed by 500 µg/ml in wich a survival observed was < 50% (42.28%). Respect to extracts applied, E3 was the most cytotoxic extract showing significant differences respect to E1 and E2. For other hand, E1 and E2 did not show significant differences between them.

The values of EC50 to E alcohol-H_2O =416.4 µg / ml, E aqueous= 634.5µg/ml, E acetone=367.3 5 µg /

ml, significant existing differences among them. In graphs 3-A, B, C the % of live organisms based on

log EC50 is indicated.



A. Alcoholic extract. B. Aqueous extract. C. Acetate extract

Figure 3 Percentage of live individuals versus concentration of extracts applied [Log (µg / ml)]

They represent the reduction of live organisms as the extract concentration increases, mainly in E3. Out of the 3 conditions used, the aqueous extract presented the lowest level of toxicity reducing to a 50% the population by reaching Log µg/mL of 2.75, different the hydroalcholic extract which reaches this value with Log µg/mL 2.62 and acetone: water Log µg/mL 2.55. When analyzing these results it can be conclude that an optimal concentration assayed for obtained extracts of blueberry used in this study is up to 367 µg/mL which matches the report by Gallegos [41] proving toxicity in blueberries in concentrations higher than 500 µg/ml. Avilés, 2016 [42] describes the toxicological properties in different natural extracts rich in phenolic compounds studied with *in vivo* model, where based on the results, reaches the conclusion that the extracts are not toxic and are safe for the consumption as therapeutic agent.

C. elegans may be used for the primary detection of new compounds like new medicine [43, 44]. In this sense, it has shown to be a robust gene model for the aging study and the stress functions, useful to reveal the existing connections between oxidative stress and lifespan [45].

For a long time, different toxicity tests with metals and metallic salts have been carried out [46, 47], proving to be a predictor of acute lethality in mammals, generating LC50 values parallel to LD50 values for the rat and mouse, since LC50 values were compared to the oral LD50 values published in mammals for the salts of the same metals [48, 49].

4. Conclusion

The concentration of total phenols, in the extracts, was greater than the flavonoid and anthocyanin content, which would indicate the presence of phenolic compounds different from anthocyanin pigments. This constitutes an advantage for the extracts under study, since the therapeutic effects of the phenolic compounds in diverse forms is related with the antioxidant activity which suggests that the activity is achieved through interactions among the diverse phenolic compounds, in addition we can highlight that the greater yield in the extracts is achieved with cheaper solvents such as the hydroalcoholic which has a greater potentiality of use in the pharmaceutical and/or dermaesthetics industry. Regarding the extracts toxicity, we could prove its safety, with the possibility to make progress in studies that allow the use of the extracts for industrial purposes. Considering the results from this study *in vitro/in vivo*, the extracts of

blueberries obtained in our region, are an important source of antioxidants to be used in a safe, non-toxic and effective way.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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