Scientific Paper

Content of anthocyanins, vitamin C and antioxidant activity in seven varieties of *Morus alba* L. fruits

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Abstract

Objective: To determine the content of anthocyanins and vitamin C, as well as the antioxidant activity of the ethanol extracts of the fruits from seven *Morus alba* L. varieties.

Materials and Methods: The content of anthocyanins and vitamin C was determined in seven varieties of *Morus alba* L. fruits, cultivated at the Pastures and Forages Research Station Indio Hatuey, as well as the antioxidant activity of the ethanol of these varieties through the phosphomolybdate radical capture essays and determination of total phenols. For the data processing a variance analysis was used. Correlations were made among the variables total antioxidant capacity, total phenols, content of anthocyanins and vitamin C, taking into consideration Pearson's correlation coefficient and two significance levels of 0,01 and 0,05.

Results: The content of anthocyanins, expressed as equivalent of cyanidin-3-glucoside, was between 40,74 and 96,07 mg/100 g, and vitamin C between 16,73 and 28,40 mg of ascorbic acid/100 mL of juice. All the extracts showed antioxidant activity. The total antioxidant capacity varied between 175,96 and 229,48 mmol of ascorbic acid/g of extract and the content of total phenols, between 291,52 and 897,17 mg of gallic acid/100 g of extract.

Conclusions: The *M. alba* fruits showed high contents of anthocyanins and vitamin C. The variety Yu-62 showed the highest concentrations of these compounds. The ethanol extracts showed high antioxidant activity, which is correlated to the high content of total phenols, anthocyanins and vitamin C present in the fruits.

Keywords: anthocyanins, vitamin C, antioxidant activity, *M. alba*

Introduction

At present, obtaining natural products constitutes an alternative source of bioactive substances for the food and pharmacological industry, due to its efficiency and low cost. The *Morus alba* L. fruits, show secondary metabolites and fundamental nutrients that stimulate their use in balanced diets (Gündeşli *et al*., 2019). Among secondary metabolites, phenolic compounds constitute the most widely utilized group in natural medicine (Sánchez-Salcedo *et al*., 2015). Anthocyanins stand out, which are accumulated in the cell vacuoles and are responsible for color in fruits and vegetables (Rodrigues *et al*., 2019). It has been proven that the high content and diversity of phenolic compounds is highly correlated with the antioxidant capacity (Kobus-Cisowska *et al*., 2020).

The presence of these compounds ascribes antidiabetic properties to fruits (Nanasombat *et al*., 2019), hypolipidemic effect (Sirikanchanarod *et al*., 2016) to prevent obesity, antitumoral, of protection against brain damage and liver-protector effects (Li *et al*., 2016), among others of high importance for animal and human health and nutrition.

In Cuba, diverse studies are referred about the use of *M. alba*. In animal husbandry, the agronomic results of this species, its benefits in milk production, parasite control, reduction of methane production, among other aspects related to ruminant nutrition, are promising. In agriculture, the studies in sheep husbandry, pig husbandry, rabbit husbandry and poultry husbandry, among others, prove the high nutritional value of *M. alba*, as an option in the feeding of these species (Peña-Borrego *et al*., 2019). In addition, works have been reported about evaluations of secondary metabolites in different organs of this plant (leaves, stems and roots) (Sande *et al*., 2016). Nevertheless, the research has been aimed at the organs of higher biomass and productivity, and not at the fruits.

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The objective of this study was to determine the content of anthocyanins and vitamin C, as well as the antioxidant activity of the ethanol extracts of the fruits from seven *M. alba* varieties.

Materials and Methods

Location. The fruit samples were collected from the germplasm bank of the Pastures and Forages Research Station Indio Hatuey (EEPFIH), located at 22º 48' and 7'' North latitude and 79º 32' and 2'' West longitude, at 19 m.a.s.l., in the Perico municipality, Matanzas province, Cuba.

The soil of this region corresponds to the lixiviated Ferralitic Red type (Hernández-Jiménez *et al*., 2015). Its topography is flat, with slope of 0,5 to 1,0 %, and has an average depth to the limestone of 1,50 m.

The seven mulberry varieties (Yu-12, Yu-62, Universidad, Acorazonada, Nueva, Cubana and Universidad mejorada) were sown in December, 2012, with planting density of 0,60 x 1,30 m. Irrigation was maintained before and after sowing. The appearance of pests and diseases was monitored each month. Fertilization was performed three times per year, with fertilizers based on N, P and K, at a rate of 300, 120 and 300 kg/ha/year, respectively. During the study no pruning was done on the plants.

Treatments and experimental design. A complete randomized design was applied and seven varieties were evaluated, which constituted the treatments.

Experimental procedure. For the essays mature fruits from these seven varieties were used. They were manually collected, in March, 2019 (1 kg) in the first hours of the morning. They were randomly selected, from plants that did not show signs of diseases or other affectation. They were put in polyethylene bags and were immediately transferred to the laboratory for their processing.

To make the ethanol extracts, successive extractions were carried out. For such purpose 10 g of fresh fruit of each variety were weighed, macerated with 150 mL of solvent (absolute ethanol) and incubated at room temperature during 24 h, with manual agitation intermittently. The ethanol mixtures were vacuum filtered and were maintained resting, in the dark, during 24 h. After the third extraction, the final filtration was concentrated to dryness in a rotary evaporator (model IKA®HB10 Basic), at temperature of 60 ºC and agitation rate of 15 rpm. The obtained extracts were stored at -4ºC until their utilization.

Quantification of total anthocyanins (TA). It was conducted by the differential pH method, described by Rapisarda *et al*. (2000) with some modifications. Ten grams of sample were weighed, which were crushed in the mortar. It was centrifuged during 15 min at 5 000 rpm, and temperature of 15 ºC. Two aliquots of two milliliters were taken from the supernatant. One was diluted in a buffer dissolution of potassium chloride (0,025 mol/L pH 1,0) and the other, in buffer dissolution of acetic acid/sodium acetate (0,4 mol/L, pH 4,5) in a 50-mL volumetric flask. After 40 min of reaction, absorbance was determined at 510 and 700 nm in a spectrophotometer (model Ray Leigh UV-2601). All the essays were conducted in triple. The result was expressed as mg/100 g of cyanidin-3-glucoside.

Quantification of vitamin C (ascorbic acid). It was done by the method described by Ciancaglini *et al*. (2001), with some modifications. The fruits were mechanically crushed, centrifuged at 8 000 rpm during 10 min, at temperature of 20 ºC and the supernatant was filtered. One milliliter of the juice filtered in a 250-mL Erlenmeyer flask was added, 60 mL of distilled water, 1 mL of 15 % HCl and 1 mL of 1 % starch, were added. It was evaluated with a previously standardized solution of iodine at 25 mM, until obtaining persistent color change (orange-blue) during 30 seconds. Each milliliter of iodine, 25 mM spent in titration, is equivalent to 8,806 mg of ascorbic acid. Each sample was done in triple. The result was expressed in milligrams of ascorbic acid/100 mL of mulberry juice.

Total antioxidant capacity by the phosphomolybdate radical capture method (TAC). The essay is based on the reduction of Mo(VI)-Mo(V) by the extracts and, consequently, on the formation of a phosphate green complex/ Mo(V) at acid pH (Umamaheswari and Chatterjee, 2008). The absorbance of the solution was measured at 695 nm against a blank in the spectrophotometer T70 UV/VIS. The results were expressed in mmol of ascorbic acid/g of extract. The tests were conducted in triple in each experiment and the result was informed as the mean ± *SD*.

Content of total phenols (TP). The concentration of phenolic compounds in the samples was determined according to the Folin-Ciocalteu method (Ocampo *et al*., 2014). The absorbance was measured at 760 nm in a spectrophotometer T70 UV/VIS. For the preparation of the calibration curve solutions of gallic acid were used, with concentrations between 25 and 400 μg/mL. The levels of phenolic compounds were expressed in equivalents of gallic acid mg/100 g of the extract. The tests were conducted in triple in each experiment and the result was reported as the mean ± SD.

Statistical analysis. A variance analysis was carried out for the data processing, after the fulfillment of the assumptions of variance homogeneity (Levene's test) and normality (Shapiro Wilk). The comparison among means was done through Duncan's multiple range test ($p \leq 0{,}05$). Correlations were made to determine the interrelation among the variables total antioxidant capacity, total phenols, content of anthocyanins and vitamin C. Pearson's correlation coefficient and two significance levels of 0,01 and 0,05 were taken into consideration. All the above-mentioned analyses were done by the statistical package SSPS® Statistics 22.0.

Results and Discussion

Quantification of total anthocyanins (TAC). The content of total anthocyanins (TAC), expressed as cyanidin-3-glucoside equivalent (mg/100 g of extract), showed significant differences among the varieties (fig. 1).

The variety Yu-62 showed the highest content of anthocyanins (96,07 mg/100 g). It was followed by Universidad mejorada and Yu-12. Universidad was the one with the lowest content of cyanidin-3-glucoside (40,74 mg/100 g).

These values are within the range reported by Lee and Hwang (2017), who proved that the content of total anthocyanins, expressed in mg/100 g equivalent of cyanidin-3-glucoside, increased from 0 to 2,0 g/100 g as the fruits matured. Likewise, they are in correspondence with those obtained by Jung *et al*. (2019), who indicate content of 75,85 mg/100 g of cyanidin-3-glucoside for fruit extracts from other *M. alba* varieties, in Korea. Nevertheless, in the same species lower values were reported than the ones shown in this study.

Aljane and Sdiri (2016) obtained anthocyanin content for *M. alba* of 1,35 mg cyanidin-3-glucoside/100 g in mulberry fruits, cultivated in arid regions of Tunisia. Likewise, there are studies that prove higher contents of anthocyanins in this species. Zhao *et al*. (2018) reported high values and increase in the total content of anthocyanins during the maturation of the fruit from *M. alba* var. cheongil until reaching 59,16 mg/g of cyanidin-3-glucoside (equivalent to 5916,0 mg/100 g). Meanwhile, the cultivar *M. alba* var. Turkey did not have presence. Both cultivars are located in the same province of Korea. In other works the lack of anthocyanins (cyanidin-3-glucoside) in *M. alba* fruits, is proven.

Sánchez-Salcedo *et al*. (2015) observed that total anthocyanins varied significantly among the *M. nigra* clones; while *M. alba* did not show anthocyanins. Krishna *et al*. (2020), in a study with 10 genotypes of *M. alba* fruits from India, observed that the content of cyanidin-3-glucoside varied for the *M. rubra* and *M. leavigata* genotypes. However, it was not found in the two studied *M. alba* genotypes.

The variation in the content of anthocyanins can be due to the existing genetic diversity among the varieties. Other factors that influence the content of phenolic compounds are the degree of maturity, analytical methods for their determination and growth conditions, such as temperature, humidity and light (Natić *et al*., 2015; Sánchez-Salcedo *et al*., 2015).

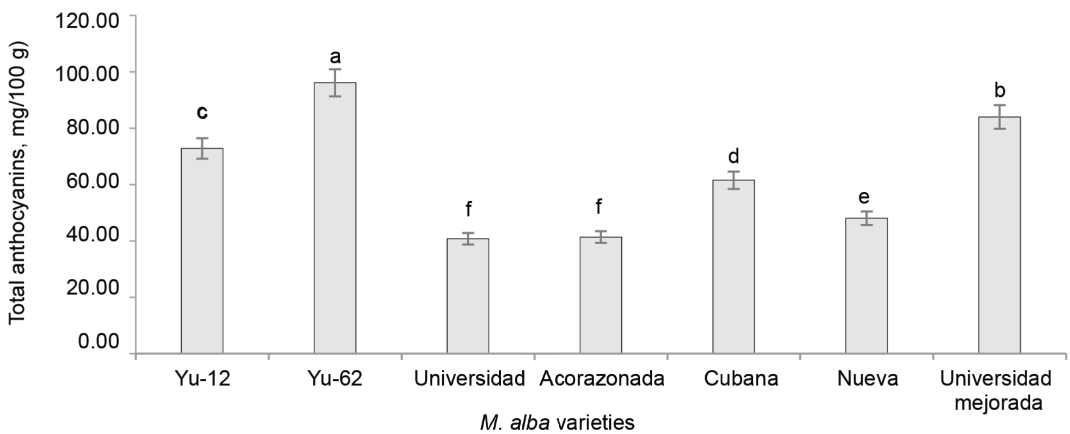

Figure 1. Content of total anthocyanins in *M. alba* fruits

a, b, c, d, e, f: different letters indicate significant differences for $p < 0{,}05$ according to Duncan

The presence of anthocyanin (cyanidin-3-glucoside) in the *M. alba* fruits reinforces the importance of *M. alba* fruits, as possible source of functional foods, because of the diverse biological and pharmacological effects they show. It is evident that the *M. alba* fruits constitute a promising source of pigments and natural antioxidants, with potentialities to be considered for their future exploitation.

Content of vitamin C (ascorbic acid). The studied varieties showed significant differences in the vitamin C contents (fig. 2). The values were between 16,73 and 28,40 mg of ascorbic acid/100 mL of juice. The variety Yu-62 was the one that showed the highest content. They were followed by Nueva, Yu-12 and Universidad. Cubana was the variety with the lowest vitamin C content.

Krishna *et al*. (2020) found vitamin C content of up to 27,10 mg of ascorbic acid/100 g of fresh weight for mulberry fruits in India. In turn, slightly lower vitamin C contents than those of the studied varieties are reported. Eyduran *et al*. (2015) analyzed the *M. alba* and *M. nigra* fruits of the Aras Valley, in Turkey, and referred vitamin C content for *M. alba* of 10,12 mg/100 g.

Gecer *et al*. (2016) achieved values in this species of 12,74 mg/100 g in the Eastern Anatolia region. Yet, Gundogdu *et al*. (2017), in a comparative study conducted between two *M. alba* varieties, from Turkey and China, found higher vitamin C content up to 31,34 mg/100 g for the cultivar from China. In similar works in the Eastern region of Turkey, Gundogdu *et al*. (2018) referred that the vitamin C content in four *M. alba* genotypes varied between 25,51 and 30,45 mg of acid ascorbic/100 g of fresh weight, values that are much higher than those in this research.

Depending on the vitamin C content, the fruit species can be classified into three groups (low, moderate and high). The vitamin C contents obtained in this study coincide with the report by Eyduran *et al*. (2015), who place mulberries among the fruit species with moderate C vitamin content.

The importance of ascorbic acid (vitamin C) is ascribed to its properties as hydrosoluble antioxidant, which favors the enzymatic activity, because it acts as cofactor in different reactions and participates in the sequestration of different oxygen reactive forms and in the reduction of free radicals. Thus, it reduces oxidative chain reactions and foresees damage on foodstuffs, besides acting in synergy with vitamin E, and preventing lipid oxidation induced by the superoxide radical (Oroian and Escriche, 2015).

Total antioxidant capacity. The obtained values indicate that all the extracts have antioxidant capacity. Significant differences were observed among the studied varieties (figure 3). The TAC showed values between 175,96 and 229,48 mmol of ascorbic acid/g of extract. The varieties Yu-12, Yu-62, Universidad and Nueva showed the highest activities, with higher values than Acorazonada, Universidad mejorada and Cubana.

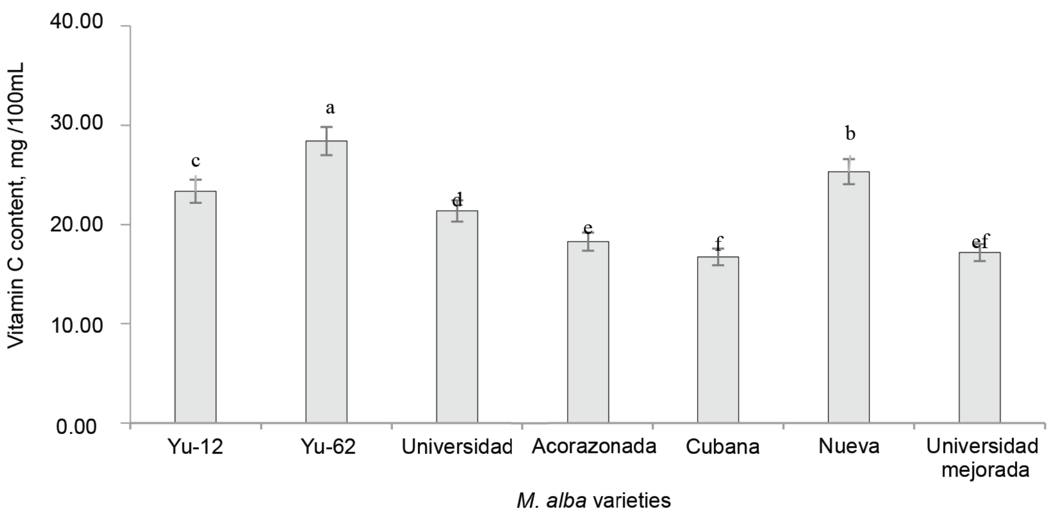

Figure 2. Vitamin C content in M. alba fruits (mg ascorbic acid/100 mL juice)
a, b, c, d. e, f: different letters indicate significant differences for $p < 0,05$ according to Duncan

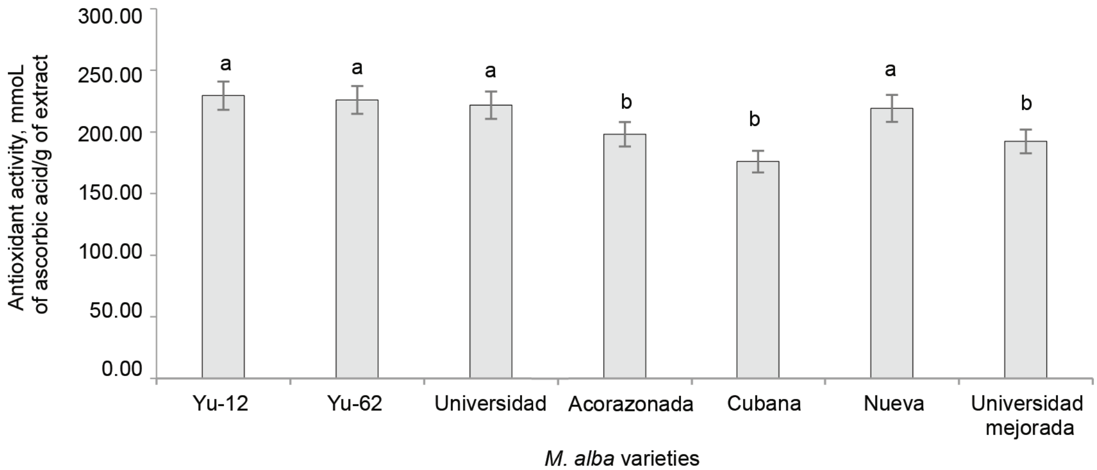

Figure 3. Total antioxidant activity of ethanol extracts in fruits from seven *M. alba* varieties.
a, b: different letters indicate significant differences for p < 0,05 according to Duncan

The total antioxidant activity shown by the fruits can be due to the presence of phytochemicals (tannins, terpenoids, steroids, saponins and flavonoids), as well as to antioxidant substances such as vitamin C (Rodrigues *et al*., 2019).

In a study of the total antioxidant activity of root extracts of 11 *M. alba* varieties and hybrids in Cuba, Sande *et al*. (2016) refer that the ethanol extract was the one that showed better total antioxidant activity in all the studied varieties. It was followed by the aqueous extract and, finally, by the hexanic one. The maximum value for the ethanol extracts of roots, determined through the phosphomolybdate radical capture essay, was much higher than the maximum value of this activity for the studied fruit extracts.

Issa and Abd-Aljabar (2013) evaluated by several methods the antioxidant activity for different extracts of *M. nigra* fruit (ethanol, flavonoid, anthocyanin and isolated pigment). These authors reported that the ethanol extract of the fruits showed higher total antioxidant capacity, evaluated by the phosphomolybdate radical essay, and was followed by the flavonoid extract. They also stated that this higher activity is due to the presence of phenolic compounds with redox properties, which allow them to act as reducing agents, hydrogen donors and oxygen eliminators in single state.

Different studies also indicate that the capacity to donate electrons of bioactive compounds is associated to the antioxidant activity, which contributes to reduce the oxidized intermediates of the lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Lee *et al*., 2015). In this research, the extracts from the evaluated varieties showed the capacity to donate electrons. Thus, they could act as terminators of the radical chain, and could transform oxygen reactive species and other free radicals into more stable non-reactive products.

Content of total phenols. The contents of total phenols (TP) of the extracts showed values between 291,52 and 897,17 mg of gallic acid/100 g of extract (fig. 4). The highest values were found in the variety Yu-62. It was followed by Universidad, Universidad mejorada, Yu-12, Nueva, acorazonada and Cubana, with significant differences among them.

The results of this study are similar to the ones referred by other authors. Jin *et al*. (2017) reported contents of total phenols (TP) between 67 and 770 mg of gallic acid /100 g of fresh weight. Meanwhile, Farahani *et al*. (2019) referred values between 134,73 and 922,64 mg of gallic acid/100 mg of fresh weight. Likewise, they are in correspondence with the ones reported by Natić *et al.* (2015) for other mulberry varieties, cultivated in northern Serbia. In other works lower TP contents than the ones in this research are referred. Lou *et al*. (2012) found that the TP content in the mulberry fruit varied from 185 to 344 mg of gallic acid/100 mg of fresh weight. These authors reported that, in the fruits with red color, phenolic compounds increase in the final stages of maturation, which is due to the accumulation of flavonols and anthocyanins.

Sánchez-Salcedo *et al*. (2015) emphasized that the genetic differences, environment and maturity

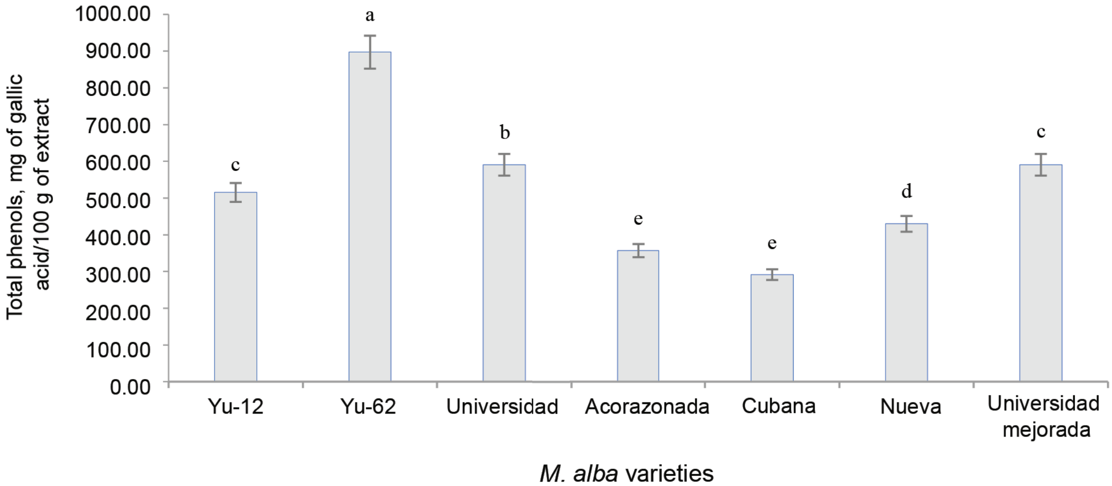

Figure 4. Contents of total phenols in the ethanol extracts from fruits of seven M. alba varieties
a, b, c, d, e: different letters indicate significant differences for p < 0,05 according to Duncan

stages influence the phenolic compounds of the fruit and the synthesis of other secondary metabolites. In addition, this variation can also be associated with the extraction methods.

Phenols are known as the compounds responsible for the smell and color of many fruits. This is due to the fact that during the maturation process many secondary metabolites, products of the metabolism of phenylpropanoids, among them pigments, anthocyanins, flavonoids and phenols, are accumulated in the vacuoles. In spite of the existing differences in the contents of total phenols of the studied varieties, the results of this research showed that the mulberry fruits could be an important source of phenolic constituents. The conduction of further studies about the phytochemical profile of these varieties would be very useful, because of the beneficial properties these components contribute to health.

The presence of phenolic compounds, including anthocyanins, flavonoids and phenolic acids, can contribute, in different form and proportions, to the antioxidant activity of fruits. Diverse studies prove the existence of high correlations of these indicators (Belwal *et al*., 2019; Rodrigues *et al*., 2019).

Correlation between total antioxidant capacity, content of total phenols, anthocyanins and vitamin C of ethanol extracts. Many studies refer correlation between the *in vitro* antioxidant activity and the content of phenolic compounds and other free-radical sequestering substances (Koss *et al*., 2019). However, Stinco *et al.* (2015) stated that the results of many variables that are correlated can vary, because this depends on several factors, such as compound type, chemical structure, synergic effects and specific applied conditions.

Regarding this, a highly significant ($p < 0,01$) and positive correlation was found between the indicators TAC and TP (r=0,621) (table 1) as well as for TAC and vitamin C content (r=0,723).

Krishna *et al.* (2020) found positive correlation between the content of anthocyanins and antioxidant capacity measured by CUPRAC (cupric ion reducing antioxidant capacity essay) of 0,95 and FRAP (ferric reducing antioxidant potency test) of 0,96. Likewise, they obtained high and positive correlation between the content of total phenols and antioxidant capacity (0,88 for CUPRAC and FRAP, respectively).

Table 1. Correlation matrix between total antioxidant capacity (TAC), content of total phenols (TP), anthocyanins and vitamin C of the ethanol extracts of *M. alba* fruits.

Indicator	TAC	TP	Anthocyanins	Vitamin C
TAC	-			
TP	0,621**	-		
Anthocyanins	0,231	0,436	-	
Vitamin C	0,723**	0,523	0,289	-

** p < 0,01

Natić e*t al*. (2015) reported high correlation between the content of total phenols and antioxidant activity, evaluated through the reducing capacity (RC) essay and the method of superoxide anion scavenging activity (SAS), with correlation of 0,838 and 0,933, respectively.

Kobus-Cisowska *et al*. (2020), in a study of the antioxidant potential and main composition of polyphenols in different extracts of *M. alba* fruits, referred that the acetone extracts showed high capacity of elimination of radicals DPPH• and positive correlation between the antioxidant effect DPPH• and total flavonoids (r=0,63), rutin (r=0,69) and ferulic acid (r=0,78). These authors state that the antioxidant activity of polyphenols confers redox properties, due to the capacity of these compounds to donate hydrogen atoms (reducing agents) in the oxidation-reduction reactions. Consequently, they can act as constituents of metallic chelates or can eliminate free radicals (Zhang *et al*., 2018).

In other species of the same genus, the existence of positive correlations between the antioxidant activity and content of flavonoids and phenolic compounds, is also reported. Hosseini *et al*. (2018) found in the study conducted with *M. nigra* L. fruits positive correlation between the content of flavonoids and phenolic compounds (r=0,94). In addition, the antioxidant activity showed positive correlations with the content of phenolic compounds (r=0,80) and content of flavonoids (r=0,80).

Among the polyphenolic compounds, phenolic acids constitute phytochemicals with marked influence on the antioxidant capacity of fruits and vegetables. These acids act as inhibitors of the generation of free radicals, because they are capable of increasing the catalytic activity of the endogenous enzymes that participate in the neutralization of radicals (Jin *et al*., 2017).

According to the results of this research, the *M. alba* varieties did not show similar performance of antioxidant activity, according to the evaluated methods. This aspect can be ascribed to the fact that each phenolic component can contribute, in different form and proportions, to the antioxidant activity, and to the fact that the correlation depends not only on the concentration and antioxidant quality, but on its interaction with other metabolites present in the extracts. However, positive correlation is proven between the studied indicators and the marked antioxidant activity that was observed in all the varieties, because of the high content of anthocyanin (cyanidin-3-glucoside) and vitamin C, as well as other secondary metabolites.

The antioxidant activity is the main defense line against free radicals, formed because of cell metabolism. The antioxidant compounds present in *M. alba* fruits can neutralize these free radicals, for which they are beneficial for human and animal health. The studied varieties contribute new forms of natural antioxidants in functional and nutraceutical foodstuffs.

Conclusions

The *M. alba* fruits show high contents of anthocyanins and vitamin C. The variety Yu-62 had the highest concentration of these compounds.

The ethanol extracts showed high antioxidant activity, which is strongly correlated to the high content of total phenols, anthocyanins and vitamin C present in the fruits, for which they constitute a beneficial natural product, which can be incorporated to any type of diet.

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Conflict of interests

The authors declare that there is no conflict of interests among them.

Authors' contribution

- Yudit Lugo-Morales. Execution of the experiments, data processing, writing and corrections of the manuscript.
- Maykelis Díaz-Solares. Research design, selection of the protocols to be worked and manuscript revision.
- Nancy Altunaga-Pérez. Execution and standardization of the research protocols.
- Inelvis Castro-Cabrera. Standardization of the used protocols and data analysis.
- Denise Sande-Santos. Execution and standardization of the research protocols.
- Jacqueline Aparecida-Takahashi: Execution, standardization of the research protocols and data analysis.

- Leydis Fonte-Carballo: Execution of the protocols and data analysis.

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