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Antioxidant evaluation of a fermented alcoholic beverage of hawthorn (*Crataegus mexicana*)

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Abstract

In nature there are antioxidant compounds that can inhibit the oxidation-reduction processes in the human organism, it is important to evaluate the role of the natural antioxidants that counteract those processes. Studies *in vitro* models have shown that the extracts of acetone derived from the shell of the hawthorn (*Crataegus mexicana*) have high antioxidant activity. After being subjected to a fermentation process, it preserves this biological property. The purpose of this work was to evaluate the antioxidant activity by colorimetric techniques as well as to identify some metabolites by HPLC-masses of the fermented beverage of hawthorn.The free radical trapping capacity of the hawthorn (*C. mexicana*), was determined using DPPH and ABTS.The fermentation was developed in two media, in natural environment and with *Saccharomyces cerevisiae.*The fermented beverage of hawthorn reached 12% Alc. Vol., The percentage of the antioxidant activity evaluated by the DPPH method reached 84 ± 3.6%, by ABTS 95.3 ± 3.3%. The metabolite identified in the fermented beverage of hawthorn has a mass ratio of *577 m/z* corresponding to Procyanidin B_2.Based on the above results, it is possible to mention that the hawthorn subjected to a biological fermentation process preserves the antioxidant activity.

Keywords: Antioxidant activity, Hawthorn (*Crataegus mexicana*), alcoholic fermentation, free radicals, fermented beverage, *Saccharomyces cerevisiae*.

Introduction

In the last years, great attention has been given in the area of free radicals, they are derived from reactive species of oxygen and nitrogen, can be generated endogenously and exogenously either by exposure to various physicochemical or pathological conditions.
A balance between free radicals and antioxidants is necessary for physiological function.

If free radicals exceed the body's ability to regulate them, begin an oxidative stress condition. Oxidative stress is defined as the exposure of living matter to various sources that produce the loss of balance between free radicals and antioxidants responsible for eliminating such chemical species, in favor of free radicals under aerobic conditions **(Konigsberg, 2008).**

To counteract the effects of free radicals, cells produce their own antioxidant system (endogenous) through enzymes; the other way is the production of exogenous antioxidants by food intake **(Kinsella *et al.*, 1993).** It has been shown in clinical studies that the consumption of natural antioxidants of fruits and vegetables reduces the oxidative stress for example: extracts of apple with high content of polyphenols, similarly with nutritional supplements rich in antioxidants **(Céspedes *et al.*, 2008).** Another example is the fruit of hawthorn (*Crataegus mexicana*) in which the antioxidant action of acetone and methanol extracts of the hawthorn shell has been evaluated as well as evaluation of the extract of hawthorn in the inhibition of lipoperoxidation in human erythrocytes **(Banderas *et al*., 2015).**

One of the most efficient antioxidant groups are flavonoids, phenolic compounds that are partof the non-energetic of the human diet. They are found in vegetables, seeds, fruits and drinks like wine and beer mainly. Although several studies indicate that some flavonoids have pro-oxidant actions, this occurs only at high doses. In most studies, anti-inflammatory, antiviral or anti-allergic effects and their protective role against cardiovascular diseases are found. An example is red wine, it has an important role in the prevention of cardiovascular diseases and some types of pathogens including cancer **(Cordova *et al.*, 2009).**Red wine is attributed antioxidant effects in human plasma, it has high levels of urate and polyphenols**(Modun *et al*., 2008).**

On the other hand, the beer also contains important amounts of flavonoids among which the polyhydroxyflavans a) catechin and b) epicatechin), anthocyanogens c) leucocyanidine d) leucopelargonidine) and flavonols (group of quercetins: e) kaempferol), f) Myrecitin) **(Roos *et al.*, 2002).** (Figure 1).

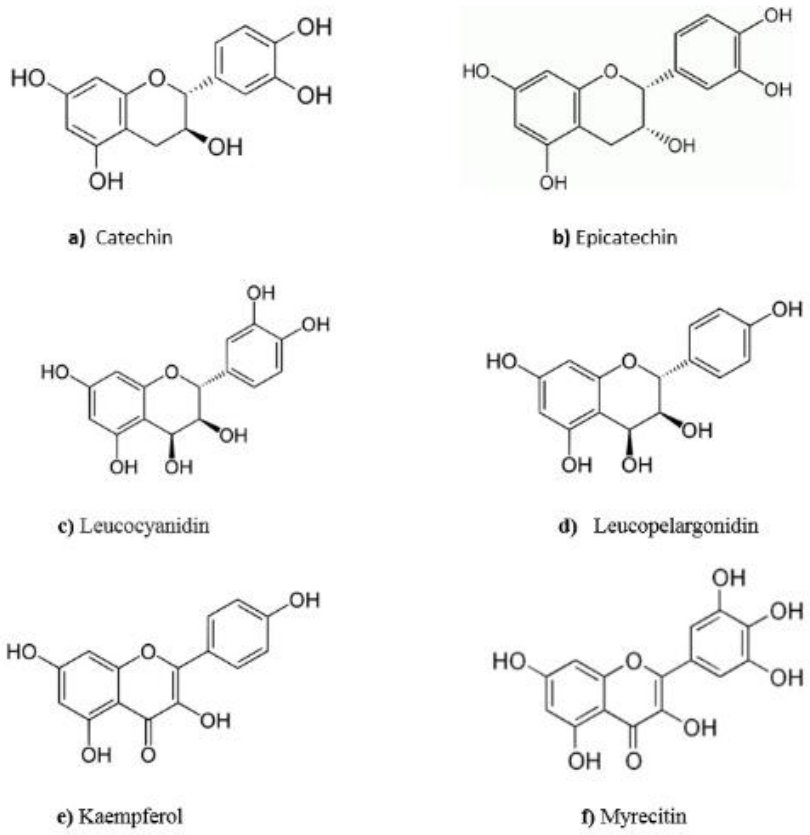

Figure 1- Main flavonoids identified in beer

In this project, the capacity of free radical entrapment and the inhibition of the lipoperoxidation of the hawthorn *(Crataegus mexicana)* was evaluated *in vitro*, hawthorn was subjected to a biological process of alcoholic fermentation for 40 days under normal conditions. The appropriate environment to develop alcoholic fermentation was also determined in artificial environment (By adding a yeast *Saccharomyces cerevisiae*) or in a natural environment (to evaluate if the fruit of hawthorn contains native yeasts that could initiate and to develop the alcoholic fermentation) and obtain a fermented drink with good taste, smell and aroma.

Finally, the polyphenol antioxidant metabolite Procyanidin B_2 was identified. In this research, it was demonstrated that the raw fruit of hawthorn (*Crataegus mexicana*) presented antioxidant activity in several models of quantification consistently, before and after fermentation. The beverage always maintained the antioxidant activity after the alcoholic fermentation process.

Materials and Methods

Chemicals and reagents

Folin-Ciocalteu reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, trichloroacetic acid, bovine serum albumin (BSA), potassium persulfate, ferrous sulfate, ethylenediaminetetraacetic acid (EDTA), sodium acetate trihydrate, phosphate buffered saline (PBS),6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2´-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt (ABTS),Deionized (MilliQ) water was used in the experiments. All of the solvents were of analytical grade.

Harvesting of hawthorn *(C. mexicana)*

The fruits were collected in December 2016 in three municipalities (Terrenate, Tepetitla and Ixtacuixtla) in Tlaxcala state, Mexico.

In the three municipalities, were collected a total of 20 kg of fruits. The selection criteria were the same size, color and pest free. Pulp and epidermis of hawthorn (*Crataegus mexicana*) was used. Then the fruit was washed and refrigerated at a temperature of -5° C.

Preparation of the fermented beverage of hawthorn *(C. mexicana)*

For the alcoholic fermentation process, two types of fermentation was used.

To begin the process of alcoholic fermentation, we used an Applikon AD1 1012 bioreactor with 110 V Motor Controller. The bioreactor used a stirring speed of 270 rpm, 22 °C, pH3, the first fermentation was at 72 hours and the second at 120 hours.

The alcohol levels were measured by anbreathalyser with a scale of 0-10 G / L, the Brix grades were measured by a digital refractometer for sucrose.

A) Alcoholic fermentation of hawthorn (*C. mexicana*) with yeast *Saccharomyces cerevisiae* (Dry Ale Yeast US 05) in artificial médium

1 kg of pulpa and epidermis of hawthorn *(Crataegus mexicana)* was macerated for 45 minutes, to extract the main metabolites and compounds of the fruit, then a pasteurization was performed, to eliminate the pathogens that can affect the sensorial characteristics of the beverage, The yeast was added at 30 °C and finally the alcoholic fermentation process begins with a period of 40 days.

To determine the degrees of alcohol, a breathalyzer with a scale of 0-10 G/L was used and to determine the degrees Brix present in the beverage was determined by a digital refractometer for sucrose.

Quantification of antioxidant activity

Inhibition of free radical DPPH

The free radical trapping capacity of the hawthorn (*C. mexicana*), was determined using DPPH [9]. A stock solution of 1mM DPPH in ethanol was prepared;

All plates of the 96-well Elisa reader were filled with 50 µL of the sample to be evaluated and 150 µL of DPPH; were incubated for 30 minutes at 37 ^{o}C with orbital agitation.

The readings were made at λ = 517 nm in a Bio-Tek EL800 microplate reader.

Formula to calculate the% reduction of DPPH = [(C-E) / C] x 100:
C = Control Absorbance
E = Absorbance of the sample to be evaluated

ABTS radical inhibition

To determine the sequestering capacity of the fermented product, the procedure described by Kuskoski[10] was followed. Consisted of producing the ABTS radical by the solution of ABTS (7,2-azinobis-3-ethylbenzothiazolin-6-sulfonic acid, diammonium salt) with 2.45 mM potassium persulfate (final concentration). It was mixed and incubated in the dark for 12-16 h at 25 ^{o}C. Once formed the radical was diluted with ethanol until an absorbance value of 0.70 to 436 nmwas obtainedin a Genesys 10 UV(Electroncorparation)spectrophotometer.10 µL of the sample was taken and 990 µL of the ABTS solution was added, the sequestering effect was monitored every minute for 6 minutes.

To determine the percent reduction the following formula is applied:

TEAC = (% reduction – 3.09777)/4.76498

Identification of bioactive metabolites present in hawthorn *(Crataegus mexicana)*

The fermented beverage of *C. mexicana* was vacuum packed in a sterile bottle with a volume of 330 mL. For the identification of metabolites an Agilent 1200 liquid chromatograph with Bruker Esquire 6000 mass spectrometer was used, nebulizer with 50 psi, drying gas of 3 L/min, drying temperature of 350 °C and pressure temperature of 400 °C, UV 287, wavelength of 254 nm, Kinex C_8 column of 2.6 µm, eluents, methanol and water (0.1% formic acid), flow rate of 0.03 mL/min and the sample was dissolved in methanol.

Results and Discussion

Fermentation of hawthorn *(C. mexicana)* in natural environment

The results obtained during the fermentation process in natural environment showed that the native microbiota of hawthorn (*C. mexicana*) is unable to degrade sucrose as a carbon source in a period of 20 days, Alcoholic fermentation process with native microbiota of hawthorn (*C. mexicana*), showed the absence of alcohol at the end of the fermentation at 120 hours. Evaluation of the antioxidant activity of the fermented beverage of hawthorn *(C. mexicana)* at 120 hours by DPPH and ABTS inhibition percentage was 34.43 ± 0.04 and 35.45 ± 0.02 respectively.

For all that, it was determined that the fruit of hawthorn lacks a yeast that is capable of fermenting the carbohydrates and obtaining ethanol and CO_2. As mentioned above, the microorganisms responsible for alcoholic fermentation are yeasts, *Saccharomyces, Schizosaccharomyces, Kluyveromyces, Brettanomyces, Kloeckera y Nadsonia* sp **(Prescott and Dunn, 1959; Moraes, 1981; Lima *et al.*, 1985; Evangelista, 1989; Souza &Queiroz, 1995).**The microorganism used in the ethanol fermentation process must have well defined characteristics, such as the ability to ferment carbohydrates with high yield, high fermentation rate, osmotolerance, ethanol tolerance, the ability to produce high concentrations of ethanol, acid tolerance, high cell viability for repeated recycling, high temperature resistance and the genetic stability of the characteristics mentioned above. It is compared with the research studies of Guifen He, where they mention that the different strains of yeast influence the characteristics and active components of the wines of *Crataegus,* five different yeasts were used, were characterized their physical properties and antioxidant capacity, showing that the Lalvin 71B yeast presented an excellent fermentation capacity **(Guifen He *et al.*, 2013)** on the other hand, in Suarez's studies, they mention that inoculated yeast strains can influence the properties and active components of wines **(Suárez, 2012).**

The fermentation in artificial medium of the tejocote fruit, kept its antioxidant activity because the raw material contains secondary antioxidant metabolites and is a natural source of phenolic antioxidants. Is associated with antioxidant activity, twenty white hawthorn genotypes were evaluated and had a higher phenolic content than other fruits (lychee fruits, peaches and strawberries**) (García- Mateos *et al.*, 2012).**Before starting the alcoholic fermentation process of the hawthorn beverage (*Crataegus mexicana*) the antioxidant activity was 84.23% for DPPH and 87.83% for TBARS. These results are similar to previous studies with respect to the antioxidant activity present in *Crataegus mexicana*, where the acetone extract at 100 ppm was the most active, in DPPH it was obtained 91.56% and 80.84% by TBARS **(Méndez *et al.*, 2013).**

Fermentation of hawthorn (*Crataegus mexicana*) with yeast *Saccharomyces cerevisiae* (Dry Ale Yeast US 05).

The results of the fermented alcoholic beverage of hawthorn*(C.mexicana)* in artificial medium with yeast *Sacccharomyces cerevisiae* (Dry Ale Yeast US 05) are in table 1.

Table 1. Process of alcoholic fermentation with yeast *Saccharomyces cerevisiae* (Dry Ale Yeast US 05).

Days	° Brix	% Alc. Vol.
0	14	0
20	7 ± 0.05	10 ± 0.04
40	6 ± 0.05	12 ± 0.05

The glucose present in the fermented beverage (° Brix) decreases from day 0 to 40. The percentage of ethanol increases as the process of alcoholic fermentation advances

Guifen He et al. they mention that the different strains of yeast influence the characteristics and active components of the wines of *Crataegus*, where they used five different yeasts and characterized their physical properties and antioxidant capacity, demonstrating that Lalvin 71B yeast had excellent fermentation capacity, on the other hand, in Suarez's studies, they mention that inoculated yeast strains can influence the properties and active components of wines **(Suárez, 2012).**

For all that it can be said that the optimal environment of alcoholic fermentation of the beverage of hawthorn *(Crataegus mexicana)* is in artificial environment, in other words, the wort was incubated with *Sacccharomyces cerevisiae* dry yeast (dry yeast Ale US 05) reaching an alcohol production of 12% vol. Alc., in addition to improving the sensory characteristics of the fermented beverage. In a similar work, the function, diversity and composition of the yeast strains that contribute significantly to the sensorial composition of the wine was evaluated, possibly due to the metabolic activity that characterizes each yeast species **(Romano *et al.*, 2003).**

Evaluation of antioxidant activity

Neutralization of free radicals by DPPH

The free radical trapping capacity was evaluated by the DPPH method, the most inportan fact was the capacity to keep the antioxidant activity, after the biological fermentation process.

The results ofDPPH test are shown in Figure 2.

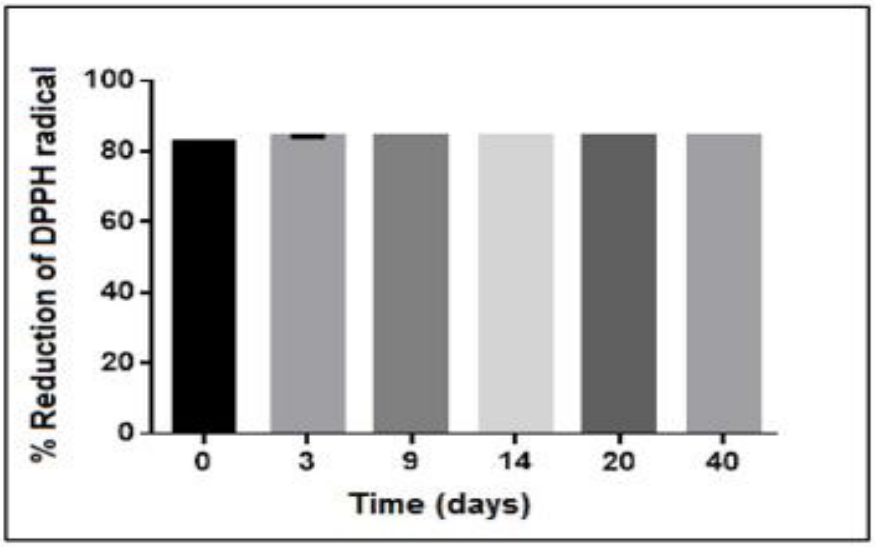

Figure 2 – Evaluation of antioxidant capacity of the fermented beverage of hawthorn (C.Mexicana) by DPPH method in artificial medicum

The results in the % reduction of DPPH radial show a siginficant differences between days 0 and 40 since P- 0.007

This is favorable, since the fermentation in an artificial medium, preserve the antioxidant activity and obtain high percentages of alcohol volume. Bonferroni's multiple comparison of the fruit of hawthorn *(Crataegus mexicana)* with yeast *Saccharomyces cerevisiae* (Dry Ale Yeast US 05), the results show a significant increase in antioxidant activity at the end of the alcoholic fermentation process.

Evaluation of the antioxidant activity of the fermented *Crataegus mexicana* neutralizing the radical ABTS

It is possible to observe the capacity of capturing free radicals by ABTS method, as well as the significant increase of this property at the end of the alcoholic fermentation process at 40 days. The capturing capacity of the fermentation is shown below, where it was compared with trolox, expressed as mg equivalent of trolox per gram of sample. (Table 2).

Table 2. Evaluation of antioxidant capacity of the fermented beverage of hawthorn *(C. mexicana)* by the ABTS method in artificial medium.

DAY	% of ABTS radical reduction	mg trolox equivalents per grams of sample
0	87.83 ± 0.003	17.78 ± 0.004
3	88.19 ± 0.006	17.81 ± 0.005
9	84.94 ± 0.028	17.85 ± 0.003
14	92.3 ± .030	18.34 ± 0.002
20	94.33 ± 0.33	18.89 ± 0.003
40	95.32 ± 0.02	19.35 ± 0.002

One-way statistical analysis with repeated measurements of the GraphPad Prism program and Bonferroni's multiple comparison. In the statistical analysis, all results show a significant increase in antioxidant activity between days 0 and 40 since P = <0.001

The results show the presence of secondary antioxidant metabolites of the polyphenolic type in the fermented beverage of tejocote (*Crataegus mexicana),*the results above is tested using more selective evaluation techniques such as ABTS since it only captures polyphenolic free radicals **(Kuskoski *et al.*, 2005).**At the end of the alcoholic fermentation process the antioxidant activity increases from 87.83% to 95.32 ± 0.02% reduction of the cationic radical. Probably the polyphenolic compounds present in the fermented beverage provide antioxidant activity, in addition to providing organoleptic characteristics to the beverage. In other investigations, the properties of phenolic compounds in grape seeds were evaluated and were found these secondary metabolites that play an important role to determine the organoleptic characteristics of berries and wines **(Robichaud *et al.*, 1990).** In particular, they contribute to wine characteristics such as color, taste, astringency and bitterness **(Robichaud *et al.*, 1990; Thorngate& Noble, 1995).**Also, the major classes of flavonoids in grapes and wines have been shown to be anthocyanins, catechins and procyanidins **(Cabanis *et al.*, 1998).**

Identification of bioactive metabolites present in hawthorn *(C. mexicana)* fermented by HPLC-Masses

Once the antioxidant evaluation stage was concluded, some secondary antioxidant metabolites were identified in the fermented beverage of hawthorn *(C. mexicana).* The results of this work in chromatography show three important peaks; 1, 3 and 7 (Figure 3)

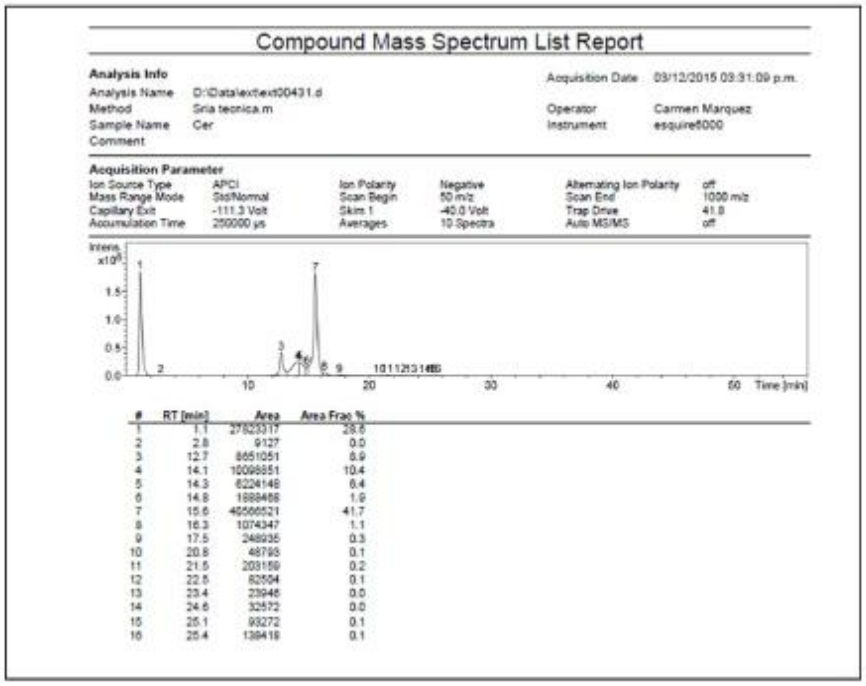

Figure 3- Chromatogram of the hawthorn fermented (*Crataegus mexicana*)

From compound 3 his mass spectrometry analysis was developed for the characterization of the secondary antioxidant metabolites (Figure 4).

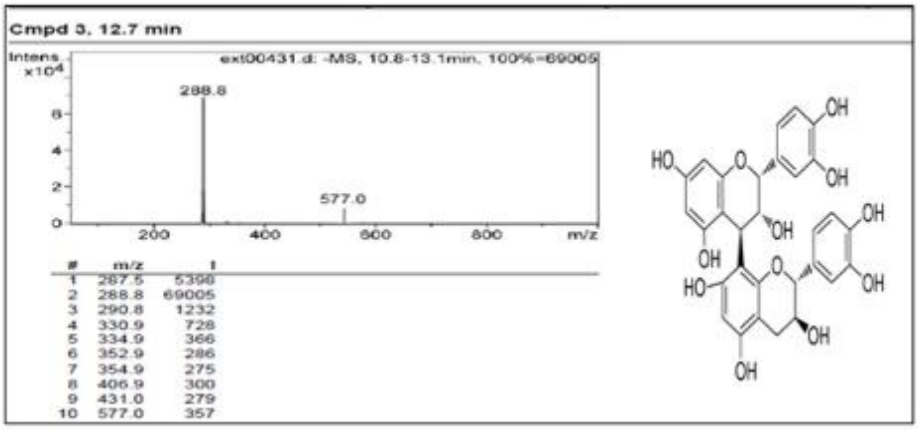

Figure 4- Mass spectrometry of the hawthorn (*Crataegus mexicana*) fermented peak.3. Compared with chemical structure procyanidin B2, molecular weight 577 g/mol

From peak number 3, a mass charge ratio of 577 *m/z* was obtained with a base peak of 288.9 *m/z*. Which, concurred with the molecular structure of procyanidin B2. Compared with LC / UV and MS/MS compilation studies, using 39 phenolic standards and elucidating metabolites present in different fermented samples, the presence of procyanidin B2 was determined **(Cooks *et al.*, 2000; Sónchez-Rabaneday*et al.*, 2003; Monagas et al., 2005; Loredana*et al.*, 2006; Stögg*let al.*, 2004).** Which had a mass/charge ratio of 577.3 *m/z* and a procyanidin B2 peak of 280 m / z. Another work that identifies procyanidin B2 in a fermented wine beverage is by means of chromatography, retention times, UV spectra and spectral UV spectral data compared to Procyanidin B2 standards. The compounds identified were gallic acid, protocatechin, flavanols, (+) catechin, (-) epicatechin and procyanidins B2, B3 and B1, among others **(Hernández *et al.*, 2008).**Possibly, the presence of procyanidin B2 in the fermented beverage of hawthorn*(Crataegusmexicana)* is due to one of the main phenolic compounds present in the fruit of

hawthorn *(Crataegus ssp.)*They are; epicatechin, aglycones and type B glycosides, oligomericprocyanidins and flavonols, phenolic acids and C-glycosyl flavones and it is estimated that the total content of phenolic compounds is higher in leaves and flowers. Procyanidins (procyanidin B2 and procyanidin B1) are known that predominate in fruits **(Baoru *et al.*, 2012).** Aother example showing the presence of the secondary metabolite in *Crataegus* is Gabriela by means of chromatographic analysis of extracts of leaves and flowers of *(Crataegus spp.)* the presence of procyanidins was revealed until a tetrameric level, this was using mainly LC-MS techniques **(Gabriela *et al.*, 2000).** Another work that characterizes Procyanidin B2 by means of seeds of grape seeds is that of Procyanidin B2, had a molecular weight of 577 *m/z* and monomers of 289 *m/z*, identified by HPLC-masses **(Jiang *et al.*, 2013).** These studies coincide with the mass spectrometry of the fermented beverage of hawthorn *(Crataegus mexicana).*

The fermented alcoholic beverage of hawthorn *(Crataegus mexicana)* obtained 12% Alc. Vol. in an artificial environment with Ale-dried yeast *(Saccharomyces cerevisiae)* in a period of 40 days of fermentation.

Conclusion

The antioxidant activity of the fermented beverage of hawthorn *(Crataegus mexicana)* was quantified by the DPPH and ABTS methods and presented an entrapment of these radicals in 90%, demonstrating that the beverage maintains the antioxidant properties of the fruit.

Regarding the process of inhibition of lipoperoxidation was calculated more than 90% efficiency.

The polyphenolic metabolite epicatechin B2 in the beverage of hawthorn *(Crataegus mexicana)* was identified by HPLC- masses.

Conflicts of interests

We declare that there is not conflict of interest in the publication of this article.

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