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Original article

Development of a quantified herbal extract of hawthorn *Crataegus mexicana* leaves with vasodilator effect

Christian Ornelas-Lim [a], Francisco J. Luna-Vázquez [a,*], Alejandra Rojas-Molina [a], César Ibarra-Alvarado [a]

[a] *Laboratorio de Investigación Química y Farmacológica de Productos Naturales, Facultad de Química, Universidad Autónoma de Querétaro, Querétaro, México*

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ABSTRACT

Hawthorn (*Crataegus spp.*) has been used for the treatment of several heart diseases and hypertension. The studies carried out on several hawthorn species have led to the development of standardized extracts useful in the cure of mild chronic cardiac diseases.

In Mexico, the most common *Crataegus* species are *C. mexicana* and *C. gracilior*. Decoctions prepared from the fruits and leaves of these species have been employed to the treat respiratory diseases, tachycardia and to improve coronary blood flow. Considering that to date there are no reports of the use of Mexican *Crataegus* species to treat cardiovascular diseases, we propose an analytical method to obtain a quantified extract of *Crataegus mexicana* leaves for the development of a standardized extract with therapeutic value in cardiovascular diseases as an alternative source to the extracts obtained from *Crataegus* species of European and Asian origin.

Therefore, the aim of this study was to obtain an extract prepared from C. mexicana leaves with the highest vasodilator activity to select the optimal chemical marker to stablish and validate a reversed-phase high-performance liquid chromatography (RPHPLC-DAD) analytical method for obtaining a quantified extract with vasodilator effect.

The results obtained from the analytical method validation, which was carried out according to the guidelines stablished in the Eurachem Guide and the ICH guidelines proved that the RPHPLC-DAD method we developed was specific, precise, accurate, and showed good linearity over the concentration range of 3 – 21 μg/ml for (-)-epicatechin and rutin, which were selected as chemical markers.

1. Introduction

Cardiovascular diseases (CVD) are the main cause of death globally. According to World Health Organization (WHO) in 2016 almost 18 million people died from CVDs, representing 31% of all global deaths (World Health Organization, 2020).

Hypertension is considered the major risk factor for cardiovascular death (Qamar & Braunwald, 2018; Yano et al., 2018) and its global trend frequency is growing, particularly in low- and middle-income countries (Forouzanfar et al., 2017) because of the high number of people undiagnosed, untreated and uncontrolled (Haldar, 2013). Therefore, it is necessary to implement new strategies in the search for new therapeutic alternatives for the prevention and treatment of hypertension.

Considering that approximately 80% of the world's population rely on plants from traditional medicine to solve their health needs (Cloud et al., 2020), especially in developing countries where many people do not have access to other health systems, standardized extracts prepared from medicinal plants could represent a very valuable alternative to address the treatment of hypertension and other cardiovascular diseases. Furthermore, in developed countries there has been an increased interest in the use of therapies of natural origin to achieve healthier and more balanced lives (Kunle, 2012).

Although herbal preparations seem to be an excellent alternative to "allopathic" drugs, it is necessary to consider that this kind of medicinal preparations could show variability in their constituents due to cultural and environmental factors such as growth, geographical location, climate, soil nutritional conditions, time of

* Corresponding autor.at: Laboratorio de Investigación Química y Farmacológica de Productos Naturales, Facultad de Química, Universidad Autónoma de Querétaro, Querétaro 76010, México.

E-mail address: francisco.luna@uaq.mx (F.J. Luna-Vázquez).

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harvesting and post-harvest storage (Tsai et al., 2015). Hence, the profile of constituents is variable from batch to batch with obvious implications in efficacy and safety (Kunle, 2012). Consequently, one of the most important features in the development of standardized herbal extracts is to determine a compound as chemical marker that guarantees a well-defined and constant composition in order to produce a drug of defined quality (Kunle, 2012).

Regarding hawthorn (*Crataegus* spp.), these species have been employed in many parts of the world for the treatment of several heart diseases and hypertension, particularly in Europe, China, and North America (Dahmer & Scott, 2010; Edwards et al., 2012; Orhan, 2016; Yang & Liu, 2012). Some *Crataegus* species have been thoroughly investigated, such as *C. pinnatifida* (Chang et al., 2002; Jurikova et al., 2012; Shao et al., 2017; Wu et al., 2014); *C. monogyna* (Bardakci et al., 2019; Barros et al., 2011; Nabavi et al., 2015), *C. laevigata* (Dahmer & Scott, 2010; Edwards et al., 2012) and *C. oxyacantha* (Orhan, 2016; Ranjbar et al., 2018; Rastogi et al., 2016; Sadek et al., 2018; Wang et al., 2013). However, since *Crataegus* is a highly variable genus, the chemical composition of its many species may significantly vary (Edwards et al., 2012; Venskutonis, 2018).

In vitro and *in vivo* studies have showed that *Crataegus* spp. extracts, obtained from the leaves, flowers and fruits, induce anti-ischemia/reperfusion-injury, anti-arrhythmic, hypolipidemic, vasorelaxant, anti-inflammatory, hypotensive and cardioprotective effects (Bujor et al., 2020; Nazhand et al., 2020; Negi et al., 2018; Ranjbar et al., 2018; Rastogi et al., 2016; Sadek et al., 2018; Shao et al., 2017; Shatoor et al., 2019; Wang et al., 2013; Wu et al., 2014). Moreover, there are evidences that hawthorn can significantly lower blood pressure in patients with mild hypertension in longer-term treatments (Cloud et al., 2020).

Studies concerning the chemical composition and pharmacological effect of *Crataegus* species have led to the development of standardized extracts and herbal medicines useful in the treatment of mild chronic cardiac diseases (Koch & Malek, 2011).

At least thirteen species belonging to the genus *Crataegus* have been identified in Mexico and the most common species are *C. mexicana* and *C. gracilior* (Banderas-Tarabay et al., 2015). All *Crataegus* species are commonly known in Mexico as *Tejocote*, a word originated from the *nahuatl* term *tetl-xocotl*, which means wild or hard sour fruit (Martínez, 1991). The fruits of these species are eaten fresh, in jam, compote or in decoctions to obtain hot beverages (García-Mateos et al., 2013). Decoctions prepared from the leaves, fruits and flowers of *Crataegus spp* have been used in Mexican traditional medicine for the treatment of several respiratory diseases such as cough, cold, bronchitis, as diuretic and to improve coronary blood flow (Arrieta et al., 2010; Hernández-Pérez et al., 2014; Martínez, 1991; UNAM, 2009).

Regarding the chemical composition of Mexican *Crataegus* species, chlorogenic acid, (+)-catechin, (-)-catechin, quercetin, kaempferol and apigenin glycosides have been identified in the ethanolic extracts obtained from the fruits of *C. stipulosa*, *C. mexicana*, *C. nelsoni* (García-Mateos et al., 2013) and *C. pubescens* (González-Jiménez et al., 2018). Additionally, ursolic, corosolic and euscapic acids have been identified in the methanolic extracts obtained from the leaves and flowers of *C. gracilior* (Hernández-Pérez et al., 2014; Torres-Ortiz et al., 2019). Both extracts showed antioxidant effect and elicited a significant vasodilator activity. The results derived from these studies indicate that *Crataegus* species growing in México could be employed for the development of phytomedicines useful to prevent and treat cardiovascular diseases in the same way as European and Asiatic *Crataegus* species.

To date there is not information about the use of Mexican *Crataegus* species for the treatment of cardiovascular diseases, therefore, in the present study we propose an analytical method to obtain a quantified extract of *Crataegus mexicana* leaves in order to develop a standardized extract from this plant as an alternative source to the extracts obtained from *Crataegus* species from Europe and Asia.

2. Materials and methods

2.1. Chemical and reagents

Gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, vanillic acid, (-)-epicatechin, quercetin, rutin, kaempferol, epicatechin gallate, catechin gallate, 2,2́-diphenil-1-picrilhidrazil, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 2,4,6-Tris(2-pyridyl)-s-triazine and the Folin-Ciocalteau reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate, sodium nitrite, aluminum chloride, sodium hydroxide, ferric chloride, sodium acetate were obtained from J.T. Baker (Mexico).

Hexane, methanol, acetone, ethanol, hydrochloric acid, acetic acid reagent grade were obtained from J.T. Baker, methanol HPLC grade was obtained from J.T. Baker (Mexico).

2.2. Experimental animals

All experimental procedures were performed in accordance with guidelines of the Mexican Official Standard NOM-062-ZOO-1999, "Technical specifications for the production, care, and use of laboratory animals" and approved by the Bioethics Committee of the Faculty of Chemistry of the Autonomous University of Querétaro.

Wistar male rats (250–300 g) were provided by the Institute of Neurobiology of the National Autonomous University of Mexico, Campus Juriquilla. Animals were housed in standard cages under controlled temperature conditions with a 12:12 h light–dark cycle. Water and food were provided *ad libitum*.

2.3. Materials and sample preparation

Four samples of *Crataegus mexicana* leaves were acquired in Querétaro, Mexico during the summers of 2016, 2018 and 2019.

2.3.1. Preparation of the crude extracts from C. mexicana leaves

Air dried *C. mexicana* leaves were ground to a fine powder and defatted through maceration with hexane for 24 h. Thereafter, the solvent was removed by rotary evaporation. Four solvent mixtures were used to obtain the respective extracts from each of the four leaf samples of *C. mexicana*: a) methanol 100% (MeOH), b) ethanol 60% (EtOH), c) acetone, water, acetic acid (AWAc, 80:18.5:1.5), and d) acetone, methanol, water, acid acetic (AMWAc, 40:40:18.5:1.5).

The extracts were prepared by sonication during 20 min of the defatted material and subsequent stirring for 24 h at 50 rpm. The material: solvent ratio was 1:10. This process was repeated three times with fresh solvent. Later, the plant material was filtered, and the solvents were removed by rotary evaporation.

2.3.2. Extraction of phenolic compounds from the concentrated crude extracts

The extraction of phenolic compounds was carried out in triplicate from the concentrated crude extracts according to Kähkönen et al. (Kähkönen et al., 1999) with some modifications: each extract sample (250 mg) was re-dissolved in 2.5 mL of its respective solvent by vortexing for 30 s and sonication for 30 min. The dissolved extracts were centrifuged for 15 min and the supernatant was transferred to a 10 mL flask. This extraction procedure was repeated three times. The extracts thus obtained were stored at −70 °C until further analysis.

2.4. Determination of the content of phenolic compounds in the extracts

The concentration of total phenolics was determined using the Folin-Ciocalteu's method reported by García-Solís (García-Solís et al., 2009). The calibration curve was prepared from 0.04 to 0.24 mg/mL with a methanolic solution of gallic acid and the content of phenolics in the extracts was expressed in gallic acid equivalent as mg of GA/g of extract.

2.5. Determination of flavonoid content in the extracts

Flavonoid content was determined by the Zhishen's method as reported by Cantín (Cantín et al., 2009). The calibration curve was prepared from 0.2 to 1.0 g/mL with a methanolic solution of (+) catechin and the content of flavonoids in the extracts was expressed in (+) catechin equivalent as mg of CA/g of extract.

2.6. Determination of the vasodilator effect using isolated rat aorta assay

The isolated rat aorta assay was employed to evaluate the vasodilator activity of the crude extracts, according to the method previously reported (Castro-Ruiz et al., 2017; Ibarra-Alvarado et al., 2010; Luna-Vázquez et al., 2018; Medina-Ruiz et al., 2019). Rats were sacrificed by decapitation (NOM-062-ZOO-1999, section 9.5.3.3). and the thoracic aorta was surgically removed and placed in a cold Krebs-Henseleit solution with the following composition (mM): 126.8 NaCl; 5.9 KCl; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 5.0 D-glucose; 30 $NaHCO_3$; 2.5 $CaCl_2$ (pH 7.4), bubbled with carbogen (95% O_2 and 5% CO_2). The intraluminal space of the aorta was rinsed with Krebs-Heinseleit fresh solution to prevent clot formation and cleaned from surrounding adipose and connective tissues. Thereafter, aorta was cut into 4–5 mm rings. These rings were mounted in 5 mL incubation chambers containing pre-warmed Krebs-Henseleit solution (37 °C) gassed with carbogen.

Tissues were stabilized for 30 min under a tension of 1.5 g at 37 °C. During this period, the bathing medium was changed every ten minutes. To stimulate the vascular smooth muscle, the tissues were contracted with 100 mM KCl, when a stable contractile tone had been reached, the bathing medium was changed every ten minutes to restore the initial resting tension.

Thereafter, aortic rings were challenged with 1 μM L-phenylephrine. The contractile force induced was defined as 100 %, and as soon as the plateau was reached, the extracts were cumulatively tested. Acetylcholine was used as positive control.

Changes in tension caused by the different concentrations of the tested extracts were detected by Grass FT03 force transducers coupled to a Grass 7D Polygraph (Grass Instrument Co, Quincy, MA, USA); they were expressed as percentages of relaxation based on the contraction generated by adding L-phenylephrine (Ibarra-Alvarado et al., 2010).

2.7. Determination of the chemical marker from C. mexicana leaves

Selection of the optimal chemical marker to establish and validate an analytical method for obtaining a quantified extract from *C. mexicana* leaves was carried out using the following strategy: a) identification of the phenolic compounds present in the different crude extracts prepared from *C. mexicana* leaves; b) quantification of the main phenolic compounds identified in the extracts and c) determination of the correlation between the concentration of each phenolic compound and the vasodilator activity of the extract. Finally, the vasodilator activity of the selected chemical marker was determined.

2.7.1. Phenolic compound profile by HPLC analysis

Performed by high-performance liquid chromatography–photo diode array detection (HPLC–PDA) using a Waters HPLC system (Millipore Corp., Waters Chromatography Division, Milford, MA, USA) consisting of a 600S controller and 2998 PDA detector. A ZORBAX ECLIPSE XDB-C18 column (150 mm × 4.5,3.5 um particle size) was used and the mobile phases were 1% formic acid in HPLC-grade water (A) and acetonitrile (B) with the following gradient conditions: a) start from time zero to four minutes (A 95% and B 5%), b) 4–7 min (A 90% and B 10%), c) 7–25 min (A 50% and B 50%), and d) 25–40 min (A 95% and B 5%) at a flow rate of 0.9 mL/ min. The injection volume was 20 μL, and the total analysis time was 40 min. The detection wavelengths were fixed as 254, 280 and 360 nm.

Identification of the phenolic compounds present in the extracts was carried out by comparison of retention times and UV spectra of chromatogram peaks with those of standards, including gallic, chlorogenic, caffeic, p-coumaric, and vanillic acids, (-)-epicatechin, epicatechin gallate, catechin gallate, (+)-catechin, quercetin, rutin and kaempferol. A calibration curve was constructed for each standard in order to quantify the phenolic compounds that could be detected in the extracts.

The extract samples used for this analysis were prepared according to the methodology described in section 2.3.2 employing 1:40 and 1:60 dilutions.

2.8. Method validation

Once the chemical marker was selected and the conditions for the HPLC analysis were determined, validation of the analytical method was carried out according to the criteria established by the Eurachem Guide: The Fitness for Purpose of Analytical Methods (Magnusson, 2014) and the ICH Guidelines (ICH, 2005). The parameters evaluated were specificity, linearity (working range), precision (repeatability and intermediate precision), and accuracy (trueness).

2.8.1. Specificity

Specificity of the method was carried out by comparing the maximum wavelengths of the standards with those maximum wavelengths obtained from *C. mexicana* leaves extract.

2.8.2. Linearity

This parameter was evaluated from seven points of calibration standards to make a calibration curve between 3 and 21 ug/mL, each concentration was analyzed in six replicates. The calibration curve was plotted representing the peak area as a function of the standard concentration. The slope (b1), y-intercept (b0), correlation coefficient (r), determination coefficient (r2), and confidence interval (IC β1) were obtained using the least squares regression method.

2.8.3. Precision

Precision was estimated by evaluating the within-day (intraday, repeatability) and between-day (interday, intermediate precision) results of analyses carried out on three different and consecutive days.

2.8.3.1. Repeatability. Repeatability was determined by calculating the percentage of relative standard deviation (% RSD) for three independent determinations at three concentrations (3.0, 12.0 and 21.0 μg/mL, for (-)-epicatechin and rutin.

2.8.3.2. Intermediate precision. Intermediate precision was carried out by calculating % RSD for three independent 3 μg/mL (-)-epicatechin and rutin solutions analyzed in three days. One-way

ANOVA was made to determine differences between everyday analysis.

2.8.4. Accuracy

Accuracy was evaluated by determining the average % recovery of the analytes (% R). This analysis was performed by adding known amounts of standard in 1:266 dilution extracts to obtain 3, 9 and 15 μg/mL concentrations. A sample of extract solution diluted 1: 266 was used as blank. Each concentration was analyzed in triplicate.

% R was calculated according to the following equation:

$$\%R = \frac{x' - x}{x\ spike}(100)$$

where x' is the value of the spiked sample, x is the value of the blank and 'x spike' is the added concentration.

Average, %R, IC β1, and variation coefficient values were calculated to determine the accuracy of the system. Acceptance criteria are %R from 98 to 102%, IC β1 including 100% and CV < 3%.

2.9. Statistical analysis

Total phenolic compounds and flavonoid content were performed in triplicate and the results were reported as the mean ± standard error mean (SEM). Analyses for identification and quantification of phenolic compounds was performed in triplicate and the results were reported as the mean ± standard deviation (SD). Statistical comparisons were carried out using one-way ANOVA with Tukey and Dunnett post hoc tests. Statistical significance was accepted within the 95% confidence limit ($p < 0.05$).

Ex vivo assays were performed in quadruplicate and the results were reported as mean ± SEM. Experimental data were fitted to a sigmoidal equation, plotted and analyzed to calculate EC_{50} and E_{max} values (GraphPad Prism 7.02, San Diego, CA, USA). These results were subjected to one-way ANOVA analysis, followed by Dunnett's post hoc test, using the statistical program GraphPad Prism 7.02.

3. Results and discussion

Numerous analytical methods have been proposed for quantifying phenolic compounds and flavonoids in herbal extracts (Bishnoi et al., 2018; Costa et al., 2015; Miguel et al., 2015; Seo et al., 2016; Suganthi & Ravi, 2019). Particularly, some techniques have been reported for analyzing phenolic compounds in *Crataegus* species (Bardakci et al., 2019; Hellenbrand et al., 2015; Koch & Malek, 2011; Orhan, 2016; Venskutonis, 2018). However, every single extract plant possesses its own complexity and variations in its chemical composition, therefore, it is necessary to identify a suitable chemical marker and develop an analytical method to quantify it in order to carry out quality control analyzes of medicinal products based on it (Souza et al., 2017).

3.1. Extract preparation

In order to find an extract from *C. mexicana* leaves that contained a high concentration of phenolic compounds and induced a significant vasodilator effect, four extracts were prepared using different solvent mixtures.

Several authors have reported that solvent systems containing acetone and acidulated water are very efficient in extracting oligomeric and polymeric procyanidins (Chen et al., 2016; Díaz-de-Cerio et al., 2017; Prior et al., 2005; Rohr et al., 2000). These types of compounds are considered responsible for the therapeutic activities attributed to *Crataegus* species. Although, other type of solvents such as aqueous methanol and aqueous ethanol have also been used in the extraction of procyanidins and other phenolic compounds (Luca et al., 2019; Rue et al., 2018; Sui et al., 2016).

3.2. Total phenolic and flavonoid contents

The results obtained from the determination of phenolic compounds and flavonoids are shown in Table 1. We found that the extracts obtained using methanol (MeOH) and acetone: methanol: water: acetic acid (AMWAc, 40:40:18.5:1.5) contained the highest yield of phenolic compounds, while the extract prepared with acetone, water, acetic acid (AWAc, 80:18.5:1.5) showed the highest flavonoid content.

3.3. Pharmacological evaluation of the crude extracts obtained from C. mexicana leaves

Several studies have shown that species of the genus *Crataegus* have a high content of phenolic compounds, particularly oligomeric procyanidins, such as catechin and epicatechin (Cloud et al., 2020; Edwards et al., 2012; Hellenbrand et al., 2015), which have pharmacological effects on the cardiovascular system (Brixius et al., 2006; Bujor et al., 2020; Orhan, 2016). In this study, we evaluated the vasorelaxant effect of the crude extracts obtained from *C. mexicana* leaves, employing the rat aorta model.

The results of the pharmacological evaluation indicated that all extracts elicited a concentration-dependent relaxation of aortic rings (Fig. 1). AWAc (E_{max} = 95.02 ± 2.89%, EC_{50} = 2.98 ± 0.10 μg/mL), MeOH (E_{max} = 100.08 ± 3.33%, EC_{50} = 3.00 ± 0.09 μg/mL), and EtOH (E_{max} = 99.98 ± 1.43%, EC_{50} = 2.99 ± 0.07 μg/mL) displayed a similar maximum vasorelaxant response, which was higher than that elicited by AMWAc (E_{max} = 85.20 ± 3.35%, EC_{50} = 14.66 ± 0.64 μg/mL).

MeOH, EtOH and AWAc were approximately three-fold more potent than acetylcholine, which was used as positive control (E_{max} = 67.61 ± 1.43% and EC_{50} = 8.67 ± 1.14 μg/mL). Moreover, these extracts obtained from *C. mexicana* leaves elicited a maximum vasorelaxant effect larger than that of this positive control.

Our research group had previously demonstrated that the methanolic extract obtained from *C. gracilior* leaves displayed a vasodilator effect and some of the phenolic compounds contained in the extract, such as chlorogenic acid, rutin, quercetin, kaempferol and (+)-catechin were identified by HPLC-DAD (Hernández-Pérez et al., 2014). These compounds have been shown to induce vasorelaxant and antihypertensive effects (Aggio et al., 2013; Novakovic et al., 2017; Patel et al., 2018; Sánchez et al., 2018; Vechi et al., 2019). Therefore, it was very likely that these secondary metabolites could significantly contribute to the vasodilation elicited by the crude extracts obtained from *C. mexicana*

Table 1
Content of total phenolic compounds and flavonoids in extracts of C. mexicana leaves obtained by using four different systems of extraction solvents.

System of extraction solvents	Total phenolics (mg of GAE/ g of dw[5])	Flavonoids (mg of CE/ g of dw[6])
AMWAc[1]	188.76 ± 13.27[b]	143.22 ± 5.87[b]
AWAc[2]	174.23 ± 16.6[a]	159.57 ± 6.54[a]
EtOH[3]	169.18 ± 3.96[a]	153.80 ± 6.45[a]
MeOH[4]	190.42 ± 11.95[b]	154.87 ± 5.94[a]

[1] AMWAc, acetone: methanol: water: acetic acid (40:40:18.5:1.5); [2]AMAc, acetone: water: acetic acid (80:18.5:1.5); [3]EtOH, ethanol (60% aqueous); [4]MeOH, methanol (100%).
[5] mg of GAE/ g of dw: milligrams of gallic acid equivalents for grams of dry weight
[6] mg of CE/ g of dw: milligrams of catechin equivalents for grams of dry weight
[a] and [b]: values in the same column followed by the same letter are not significantly different ($p < 0.05$)

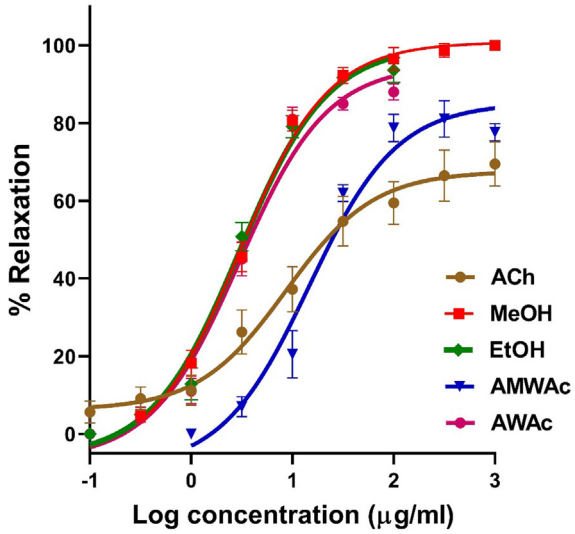

Fig. 1. Concentration-response curves of the vasodilator effect induced by the crude extracts obtained from *C. mexicana* leaves: AMWAc, AcMA, EtOH, MeOH (abbreviations are described in section 2.3.1); ACh: acetylcholine (positive control).

leaves, especially considering that these compounds have been identified in fruits and leaves of other Mexican *Crataegus* species (Banderas-Tarabay et al., 2015; García-Mateos et al., 2013).

Taking into account the content of phenolic compounds and the significant vasodilator effect of the methanolic extract obtained from the leaves of *C. mexicana*, we assumed that this extract could be used for the development of a standardized herbal extract with beneficial effects on the cardiovascular system.

3.4. Identification of phenolic compounds by HPLC-DAD

Three phenolic compounds were identified, by comparing their retention time and maximum absorbance wavelength (200 to 400 nm) with those of standards: chlorogenic acid, (-)-epicatechin and rutin. All of them, had been previously identified in the leaves, flowers and fruits from other species of *Crataegus* and had shown beneficial effects on the cardiovascular system (Bujor et al., 2020; Cloud et al., 2020; Koch & Malek, 2011; Orhan, 2016; Venskutonis, 2018).

Fig. 2 shows the chromatogram obtained from the methanolic extract compared with a standard mix chromatogram (chlorogenic acid, caffeic acid, (-)-epicatechin, rutin, hyperoside, quercetin, and kaempferol). Retention times were 14.1, 16.08 and 17.79 min for chlorogenic acid, (-)-epicatechin and rutin, respectively. The wavelengths of maximum absorption were 240 and 326 nm for chlorogenic acid, 242 and 278 nm for (-)-epicatechin, and 254 and 354 nm for rutin.

These results indicated that these chromatographic conditions did not allow a good resolution for chlorogenic acid. Moreover, the peak corresponding to rutin was not properly separated and showed a baseline variation. Therefore, considering that the wavelengths of maximum absorption for rutin are 254 and 354 nm, the chromatograms were recorded using these wavelengths and, as expected, a good resolution was obtained for this flavone. Since the baseline was not stable at 254 nm, we selected the 354 nm wavelength for rutin quantification. These same chromatographic conditions were adequate for (-)-epicatechin.

Considering that one of the criteria for selecting a chemical marker in a plant extract is the fact that this compound is responsible for the pharmacological effect elicited by the extract (Ding et al., 2017; Kunle, 2012; Ong, 2004), we chose (-)-epicatechin and rutin as potential chemical markers of *C. mexicana* leaves extracts. In order to identify these phenolic compounds in specimens of *C. mexicana* from different collections, methanolic and ethanolic leaf extracts obtained from two different years (2018 and 2016) were analyzed in addition to the extracts prepared from *C. mexicana leaves* (batch 2019).

Fig. 3 shows the chromatograms of the extracts obtained from *C. mexicana* leaves collected in 2016, 2018 and 2019. (-)-epicatechin and rutin were identified in all the analyzed extracts. Considering the presence of both compounds in all *C. mexicana* leaves extracts and their previously demonstrated vasodilator effect (Fraga et al., 2018; Kluknavsky et al., 2020; Maaliki et al., 2019; Sharma et al., 2013), we propose (-)-epicatechin and rutin as chemical markers for *C. mexicana* leaves extracts.

3.4.1. Quantification of (-)-epicatechin and rutin

In order to determine the quantity of (-)-epicatechin and rutin present in the extracts obtained from the three analyzed batches of *C. mexicana*, we obtained the calibration curves for both compounds.

The calibration curves showed good linearity in the range from 3 to 21 μg/mL (Table 2.). Thereafter, we quantified the content of

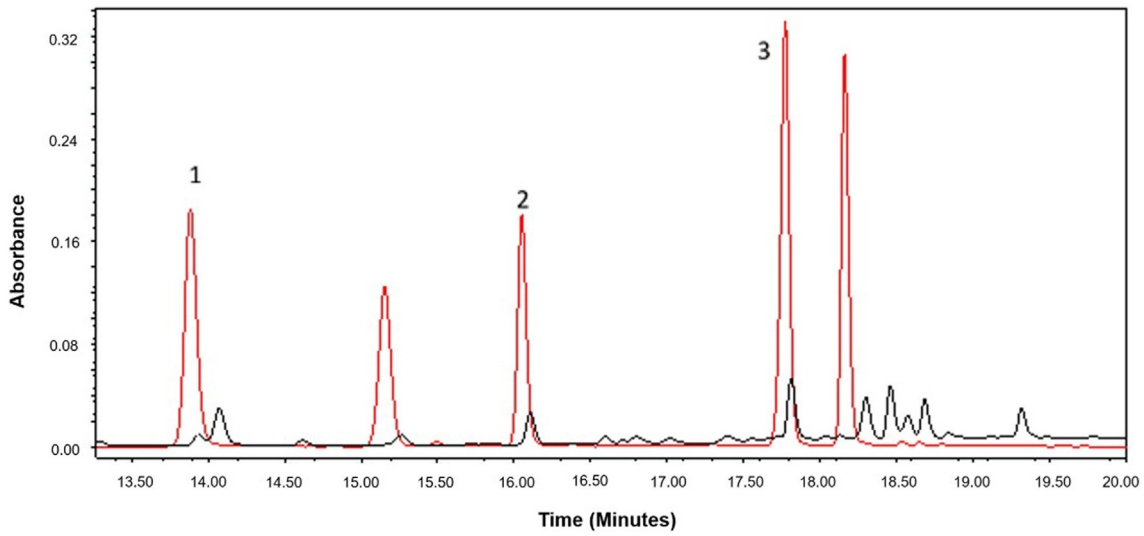

Fig. 2. HPLC-DAD chromatograms obtained from standard mix (red) and the methanolic extract of *C. mexicana* leaves (black). 1) Chlorogenic acid 2) (-)-epicatechin 3) Rutin.

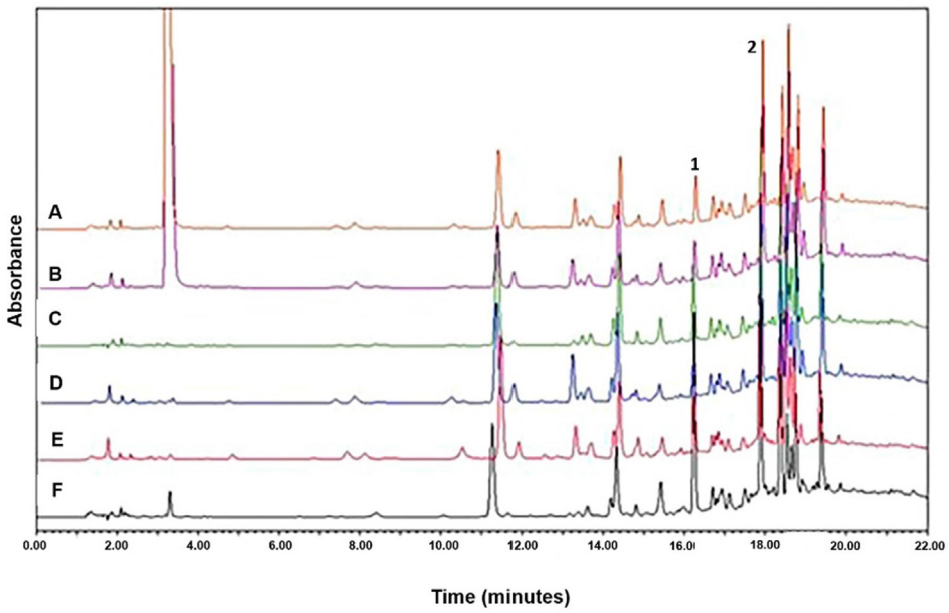

Fig. 3. Chromatograms obtained at 280 nm from AWAc(a), AMWAc (b), EtOH (c), MeOH (d) (abbreviations, see the text) extracts from *C. mexicana* leaves batch 2019, ethanolic extract and methanolic extract obtained from *C. mexicana* leaves batches 2018 (e) and 2016 (f). 1) (-)-epicatechin 2) Rutin.

(-)-epicatechin and rutin in AWAc, AMWAc, EtOH, and MeOH obtained from *C. mexicana* leaves (batch 2019) and in the methanolic extracts obtained from *C. mexicana* leaves (batches 2016 and 2018). Fig. 4 shows that all evaluated extracts have quantifiable amounts of both flavonoids. It was found that the methanolic extracts (batches 2019 and 2018) had the largest content of rutin and (-)-epicatechin. The cardiovascular activity previously reported for these flavonoids (Bujor et al., 2020; Cloud et al., 2020; Orhan, 2016; Venskutonis, 2018) and the vasorelaxant activity elicited by the methanolic extract of *C. mexicana* leaves, support the choice of this extract for the validation of an analytical method for the development of a standardized extract of *C. mexicana* leaves potentially valuable in the treatment of cardiovascular diseases.

The results indicated that methanolic extract presented the largest content in (-)-epicatechin and rutin, hence this extract was used in the validation of analytical method to develop a *Crataegus mexicana* leaves quantified extract.

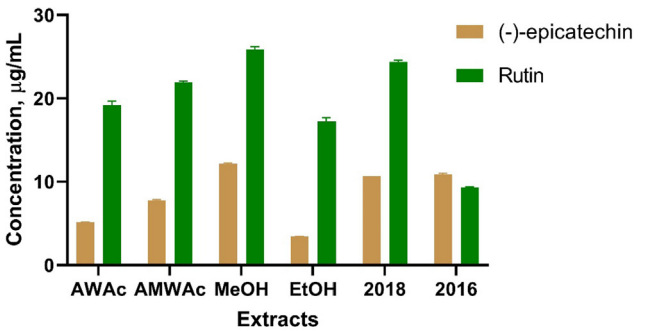

Fig. 4. Content of (-)-epicatechin and rutin in the different extracts evaluated obtained from three different batches of *Crataegus mexicana* leaves.

3.5. Validation of the analytical method for quantification of rutin and (-)-epicatechin

3.5.1. Specificity

Specificity of the method was verified by comparing the wavelengths of maximum absorption between the standards (242 and 278 nm for (-)-epicatechin and 253 and 354 nm for rutin) and *C. mexicana* leaf extracts.

3.5.2. Linearity

Linearity of the method was demonstrated by evaluating slope, correlation and determination coefficients and confidence interval (β_1) obtained by linear regression analysis of data. The statistical data of linearity agreed with ICH guidelines for analytical methods (Table 2).

3.5.3. Precision

Table 3 shows the repeatability results for (-)-epicatechin and rutin evaluated at three different concentrations (3, 12 and 21 µg/mL) for three replicates. In all cases, % RSD was within acceptance criteria (<2.5%).

Results of inter-day variability corresponding at 3 µg/mL measured at three different days are shown in Table 4. for (-)-epicatechin and rutin. According to the obtained % RSD (<3.0%), all results were under the limit as per recommendations of the International Conference for Harmonisation (ICH) guidelines. However according to the statistical analysis (one-way ANOVA), even though there was no significant difference among the

Table 2
Analytical parameters for the calibration curves obtained for quantification of (-)-epicatechin and rutin standards.

Parameter	Acceptance criteria	(-)-epicatechin	Rutin
Equation	y = mx + b	y = 15940*x + 7978	y = 36939*x + 4175
Slope	b1 ≠ 0	15940 ± 104.10	36939 ± 257.50
Correlation coefficient	r ≥ 0.99	0.9991	0.9990
Determination coefficient	$r^2 \geq 0.98$	0.9983	0.9981
Confidence interval (β1)	IC (β1) no zero included	15730–16151	36,418 – 37,459

Table 3
Results of repeatability for the quantification of (-)-epicatechin and rutin standards.

Concentration (μg/mL)	(-)-epicatechin			Rutin		
	Mean peak area	SD[1]	% RSD[2]	Mean peak area	SD	% RSD
3	52,762	912	1.729	102,837	1813	1.7635
12	198,519	3512	1.770	451,593	7037	1.558
21	335,043	6511	1.943	773,467	14,656	1.895

[1] Standard deviation
[2] Deviation standard relative percentage (coefficient of variation)

Table 4
Results of inter-day variability for (-)-epicatechin and rutin at 3 μg/mL.

	(-)-epicatechin			Rutin		
Day of analysis	1	2	3	1	2	3
Intraday mean peak area	55,563	52,552	54,567	102,401	105,704	102,566
Intraday % RSD[1]	0.99 %	2.05 %	1.48 %	0.80 %	1.55%	1.34 %
Interday mean	54,228			103,557		
Interday % RSD	2.78%			1.95%		
p (one way ANOVA)	0.03			0.08		

[1] Deviation standard relative percentage (coefficient of variation).

inter-day analysis for rutin, there was significance difference in the inter-day analysis for (-)-epicatechin. This last result may be due to variations in room temperature recorded during the different days of the test since the equipment does not have a temperature controller for the chromatographic column.

3.5.4. Accuracy

According to the ICH Q2(R1), the accuracy of an analytical method refers to the closeness of agreement between the accepted reference value and the value found and could be reported as the percent recovery of a known amount of analyte added to the sample (ICH, 2005).

In order to evaluate the accuracy of this method, (-)-epicatechin and rutin standards were added at three concentrations (3, 9 and 15 μg/mL) to the methanolic extracts of *C. mexicana* leaves, which were then analyzed using the proposed HPLC method. The recoveries of the added standards were from 97.26 to 101.73% for (-)-epicatechin and from 97.30 to 101.99% for rutin, with a variation coefficient of 2.04% and 2.07 for (-)-epicatechin and rutin, respectively. IC β1 parameters were 0.9931 – 1.0070 for (-)-epicatechin and 0.9789 – 1.0170 for rutin. These results show that interferences by other matrix components are not significant, and the HPLC conditions are suitable to obtain an adequate method accuracy.

4. Conclusions

In this study, a quantified methanolic extract with vasodilator activity was developed from *Crataegus mexicana* leaves. (-) - epicatechin and rutin were selected as chemical markers, considering their pharmacological activity and their presence in *C. mexicana* specimens collected in different years. Additionally, an analytical method, using HPLC-DAD, was developed and validated for identification and quantification of the chemical markers. The results derived from this work constitute the bases for obtaining a standardized extract from *C. mexicana* leaves, which could be employed for the development of herbal medicinal products useful for the treatment of cardiovascular diseases.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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