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Flavonoids quantification in *Acer negundo* L., extracts by HPLC analysis

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ABSTRACT

Objective: The identify and quantify, by high performance liquid chromatography, flavonoids from leaf and stem extracts of *Acer negundo*.

Design/methodology/approach: Ethanolic extracts of *Acer negundo* were analysed with high performance liquid chromatography to quantify and identify their major antioxidant flavonoids.

Results: Leaf extracts had high concentrations of rutin (34.19 μg/mL) and catechin (33.97 μg/mL), intermediate concentrations of apigenin (19.05 μg/mL), gallic acid (19.04 μg/mL), ferulic acid (17.2 μg/mL) and 2.5 dihydroxybenzoic acid (12.72 μg/mL), and low concentrations of caffeic acid (6.15 μg/mL), quercetin-3-β-glucoside (4.97 μg/mL) and isorhamnetin (4.68 μg/mL). In the stem's extracts, the highest concentrations were of ferulic acid (7.96 μg/mL), rutin (5.61 μg/mL) and catechin (4.37 μg/mL); medium concentration were identified for isorhamnetin (3.31 μg/mL) and quercetin-3-β-glucoside (2.01 μg/mL) and apigenin (0.79 μg/mL) was identified at the low concentrations. Gallic acid, caffeic acid or 2,5-dihydroxybenzoic acid were not detected.

Limitations/implications: Some flavonoids have been identified in other *Acer* species but have not been identified and quantified in *Acer negundo*, a Mexican species.

Findings/conclusions: For the first time we report gentisic acid in *Acer negundo* leaf extracts. This analytical method can be standardized to serve as a quality analysis of maple tree products.

Key words: ferulic acid, gentisic acid, flavonoids, HPLC.

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INTRODUCTION

The genus *Acer* belongs to the family Aceraceae and the order Sapindaleae, comprising 180 species (Li *et al.*, 2010). Maples (*Acer* spp.) are important in the northern hemisphere, in regions of East Asia, North America and Europe (Glensk *et al.*, 2009). *Acer negundo* is used in reforestation programs. The sap is a component that acts as a sugar source. It is currently an endangered species. The reported pharmacological activities of *Acer* species are

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antioxidant (Zhang *et al.*, 2014), antitumor (Kim *et al.*, 2015), anti-inflammatory (Ko and Choi, 2015), antibacterial (Maisuria *et al.*, 2015), antifungal, antiviral (Song *et al.*, 2015), antihyperglycemic (Zhang *et al.*, 2015), hepatoprotective (Yoo *et al.*, 2011), antiobesity (Gao *et al.*, 2012) and osteoblastic differentiation stimulator (Ha *et al.*, 2014). There are studies of compounds isolated from *Acer* showing antidepressant, skin-protective, neuroprotective, vasorelaxant, antihypertensive and antimutagenic properties. From the maple trees maple syrup is produced, an inexpensive, highly commercial product containing carbohydrates (glucose, fructose, sucrose and high molecular weight polysaccharides), minerals (Al, Ca, Fe, K, Mg, Mn, Na, and Zn), vitamins (niacin, riboflavin, and thiamine), amino acids (arginine, proline, and threonine) and organic acids (fumaric acid, and malic acid) (Zhang *et al.*, 2014). Many highly bioactive compounds have been extracted from *Acer* species, such as flavonoids, tannins, alkaloids, terpenoids, and phenolic compounds such as lignans, phenolic acids, stilbenes, and coumarins.

The chemical components with the highest bioactivity extracted from the *Acer* genus are benzoic acid derivatives (5.4%), diarylheptanoids (8.8%), simple phenolic compounds (9.7%), terpenoids and phytosterols (9.7%), tannins (12.4%), flavonoids (18.7%), phenylpropanoids (22.4%), among other compounds such as alkaloids and phenylethyl glycosides (Bi *et al.*, 2016). The most characteristic compounds existing in *Acer* species are flavonoids and tannins (Liu *et al.*, 2013). The *Acer* genus also contains important phytochemical compounds such as triterpene saponins (Glensk *et al.*, 2009). Some 331 chemical compounds have been identified from 34 species of the *Acer* genus (Bi *et al.*, 2016). However, most of the compounds from *A. negundo* have not yet been reported.

High Performance Liquid Chromatography (HPLC) is well suited for both qualitative and quantitative monitoring of various trees and has been widely used to evaluate tree and medicinal resources. Therefore, in the present research, HPLC analysis was used to analyze flavonoids from *A. negundo* extracts. There are research reports on the identification of flavonoids in *Acer* species, but do not record their quantification. Therefore, the present work reports the quantification of flavonoids obtained from renewable sources of *A. negundo* trees (leaf and stem extracts) by high performance liquid chromatography analysis, so that they are used as marker compounds for chemical evaluation or standardization of *A. negundo* and its products.

MATERIALS AND METHODS

Standards and reagents. The solvents used for the extraction and high performance liquid chromatography procedures were HPLC and analytical grade, respectively, and obtained from Sigma-Aldrich (St Louis, MO, USA). All stock solutions, standards, samples, solvents, and reagents were filtered through 0.20 μm PTFE membrane filters (Phenomenex, USA) prior to separation or injection into the instrument.

Sample collection. Stem and whole leaves of *A. negundo* were collected during autumn (October-November) 2019 in the vicinity of the city of Morelia, State of Michoacán, Mexico (19° 46′06” N 101° 11′22”′ W, 1920 masl).

Obtaining the extracts. Hundred mg samples of dried leaves and 100 mg of dried stems of *A. negundo* were taken by triplicate, these organs are renewable sources so as not

to damage the trees. The leaves and stems of *A. negundo* were macerated in a mortar with a pistil. In a 250 mL flask, 100 mL of 80% ethanol were added to each sample, the mouth of the flask was covered with aluminum foil, and each sample was allowed to rest for 24 h. The samples were then filtered on filter paper. Finally, a rotary evaporator (Buchi brand) was used to evaporate the solvent from each extract. The crude extracts were placed in amber bottles for further analysis.

HPLC analysis. The profile of phenolic compounds was determined from the methods modified by Espinosa-Alonso *et al.* (2006) and Valdez-Morales *et al.* (2014), with some modifications. Flavonoids and phenolic compounds were identified and quantified on an automatic injection chromatograph model Ultimate 3000, Dionex brand, equipped with a quaternary pump and a diode array detector. An Acclaim 120, C18 column (4.6 mm × 250 mm, 5 microns, Thermo Sci brand) was used. The mobile phase used consisted of A = acidified water to pH 2.8 with acetic acid and B = acetonitrile, a gradient was used starting with 90% A and 10% B up to 2.5 min, gradually increasing the percentage of B: 12% at 6 min, 23% at 18 min, 35% at 24 min, 95% at 30 min and returning to the initial conditions of 90% A in a final time of 40 min.

The rest of the chromatographic conditions are summarized: flow rate of 0.3 mL/min, injection volume of 10 μL, and the recorded wavelengths were 260, 280, 300, 320, 350, 375 nm. The compounds were identified by comparing their retention times and absorption spectra with the previously run standards and with which the calibration curves were made. The Chromeleon 7.0 software was used for the chromatographic analysis. The concentration values of each phenolic compound were calculated from the area under the signal curve observed in the chromatogram.

Compounds were identified as a function of their retention time and absorption spectra. Identity was only assigned to signals with a purity greater than 980 (1000 being the maximum value). The areas at the wavelength of maximum absorption of each compound were captured, as was the corresponding standard curve used. Flavonoid content was analyzed with a mean comparison test ANOVA ($P<0.05$), in the SAS statistical software (v. 2018).

RESULTS AND DISCUSSION

HPLC analysis. The isolated flavonoids from *A. negundo* extracts and identified by chromatographic analysis are shown in the chromatograms in Figure 1 and 2.

The *Acer* genus is characterized by the biosynthesis of phenolic compounds such as lignans, phenolic acids, stilbenes, coumarins and various flavonoid subclasses. Thirty-one phenolic compounds have been reported for the genus *Acer* (Bi *et al.*, 2016). The phenolic group in flavonoids directly acts by capturing missing electrons from Reactive Oxygen Species (ROS), generating less reactive species. Flavonoids act as buffers, capturing free radicals that generate the less reactive flavin radical, as the missing electrons are delocalized in it. Flavonoids can prevent the cancer occurrence by acting as natural antioxidants that prevent damage to cellular DNA caused by ROS or carcinogens. Flavonoids, both glucosides and glycosides, are also important antioxidant compounds (Wijeratne *et al.*, 2006). Secondary plant compounds, such as flavonoids

and other phenylpropanoid derivatives, act as attractants and deterrents to potential insect herbivores (Harborne and Williams, 2000). Phytochemical studies have also been conducted to investigate the phytochemical content in *Acer* species wood. Japanese maple bark has been investigated for its anticancer, anti-inflammatory, antifungal, and antibacterial effects. The bark is used in traditional Japanese medicine to treat liver disorders. Compounds isolated from *A. nikoenses* bark were catechin, rhododendrol, centrolobol, acerogenin A, B, D, K, and M, and acerides I, II, and IV (Li and Seeram *et al.*, 2011).

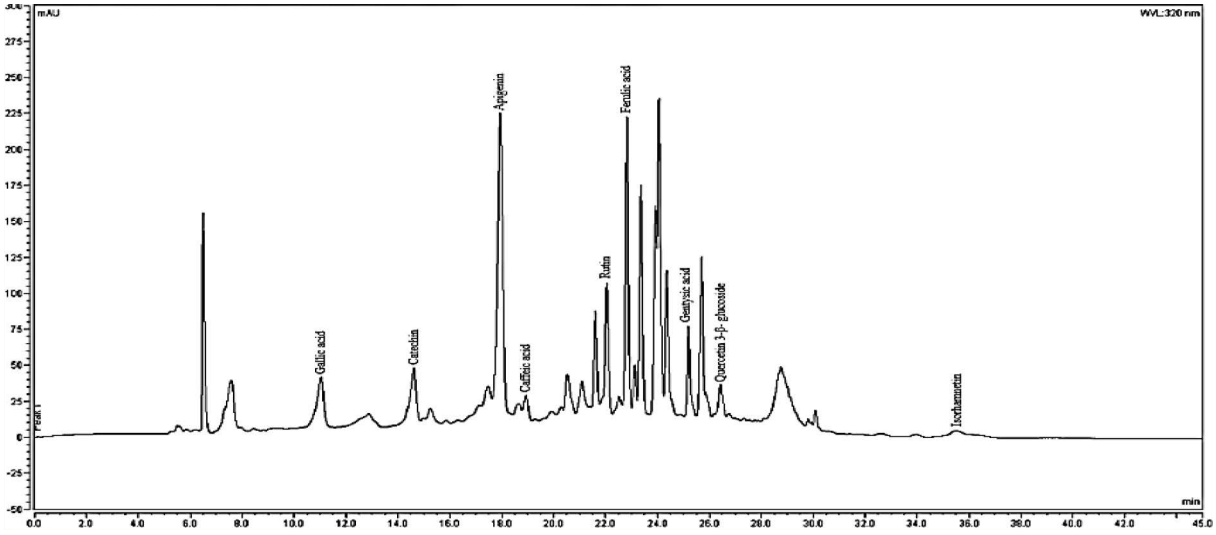

Figure 1. Compound identification by HPLC from leaves of *A. negundo*. Chromatograms of flavonoids, which were obtained from ethanol extract of *A. negundo* by column chromatography. Gallic acid ($C_7H_6O_5$), catechin ($C_{15}H_{14}O_6$), apigenin ($C_{15}H_{10}O_5$), caffeic acid ($C_9H_8O_4$), rutin ($C_{27}H_{30}O_{16}$), ferulic acid ($C_{10}H_{10}O_4$), gentisic acid (2,5-dihydroxybenzoic acid; $C_7H_6O_4$), quercetin 3-β-glucoside ($C_{21}H_{20}O_{12}$), and isorhamnetin ($C_{16}H_{12}O_7$).

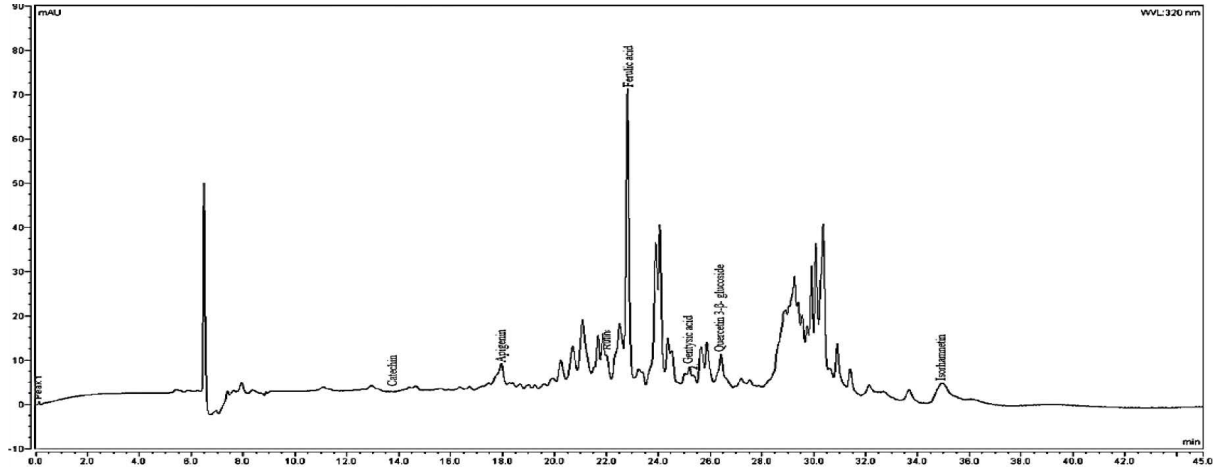

Figure 2. Compound identification by HPLC in *A. negundo* stems. Chromatograms of flavonoids, which were obtained from the ethanol extract of *A. negundo* by column chromatography. Catechin ($C_{15}H_{14}O_6$), apigenin ($C_{15}H_{10}O_5$), rutin ($C_{27}H_{30}O_{16}$), ferulic acid ($C_{10}H_{10}O_4$), quercetin 3-β-glucoside ($C_{21}H_{20}O_{12}$), and isorhamnetin ($C_{16}H_{12}O_7$).

Understanding the differences between the compounds of different species of the *Acer* genus brings us closer to the chemotaxonomic distribution of the compounds. In *A. campestre* wood, cellulose, pentosan/xylan and lignin contents were determined with HPLC (Antczak *et al.* 2013).

1) Gallic acid. Zhang *et al.* (2015) identified gallic acid in leaf extracts of *A. pseudoplatanus* by nuclear magnetic resonance. Gallic acid (tannin) is also present in the stem, leaf and bark of *A. barbinerve*, *A. tataricum*, *A. negundo*, *A. platanoides*, *A. rubrum*, *A. truncatum*, *A. pentapomicum* (Dong *et al.*, 2006). In addition, many galloyl-containing flavonoid glycosides have been isolated from the leaves of *A. tataricum* subsp. ginnala (Maxim.) Wesm., *Acer okamotoanum*, *A. rubrum* L. and *A. platanoides* L., and two glycosides exhibited strong inhibitory activity against HIV-1 integrase (Kim *et al.*, 1998). Among these flavonoids, anthocyanins have been of great interest because they are responsible for the leaf color change in spring and autumn. Cyanidin-3-(2",3"-digalloyl-β-glucopyranoside) was the first example of a di-acetylated anthocyanin with gallic acid (Fossen and Andersen 1999). In our research we found gallic acid in leaf extracts (19.04 μg/mL) but not in stem extracts.

2) Catechin. Nugroho *et al.* (2015) analyzed three phenolic substances (salidroside, catechin and scopoletin) by HPLC analysis from three methanolic extracts of stem bark, heartwood and leaves of *A. tegmentosum*. The major metabolite produced from the methanolic extract of stem bark was: salidroside: 80.22 mg/g, and in lower concentration catechin: 23.31 mg/g, and scopoletin 9.45 mg/g. Catechin was identified in extracts of stems, stem bark, wood and bark of *A. barbinerve*, *A. mandshuricum*, *A. maximowiczianum*, *A. rubrum*, *A. tegmentosum* (Lee *et al.*, 2014). In our research, we found catechin in leaves (33.97 μg/mL) and stems (4.37 μg/mL) of *A. negundo* extracts.

3) Apigenin. In leaf extracts of *A. palmatum* (Aritomi, 1963) and *A. oblongum* (Parveen *et al.*, 1988) the flavonoid apigenin was found. In our research, we found apigenin in the extracts of leaves (19.05 μg/mL) and stems (0.79 μg/mL) of *A. negundo*.

4) Caffeic acid. Caffeic acid was identified in dormant shoots of *A. saccharum* (Thakur, 1977). Here, caffeic acid was identified in *A. negundo* leaf extracts (6.15 μg/mL) but not in stem extracts.

5) Rutin. The flavonoid rutin was found in both, leaves and stem bark of *A. tataricum* subsp. ginnala, *A. glabrum*, *A. rubrum* and *A. negundo* (Backheet, 2003). In our study we found rutin in the leaf (34.19 μg/mL) and stem (5.61 μg/mL) extracts of *A. negundo*.

6) Ferulic acid. In dormant shoots of *A. saccharum* they ferulic acid has been identified (Thakur, 1977). In this research we found ferulic acid, a phenylpropanoid, in extracts from the leaves (17.2 μg/mL) and stems (7.96 μg/mL) of *A. negundo*.

7) Gentisic acid (2,5-dihydroxybenzoic acid). As hydroquinone, gentisic acid easily oxidizes and is used as an antioxidant excipient in some pharmaceutical preparations (Kostiuk *et al.*, 1988). In this research, ferulic acid was identified in *A. negundo* leaf extracts (12.72 μg/mL) but not present in stem extracts.

8) Quercetin 3-β-glucoside. Quercetin has anti-ulcer properties by protecting the gastric mucosa (de Lira *et al.*, 2009). Ma *et al.* (2005) isolated quercetin-3-O-L-rhamnoside from *A. truncatum* by HSCCC (High-Speed Counter-Current Chromatography) type chromatography, the analysis was based on studying an ethyl acetate extract of the leaves.

We obtained 41.9 mg of quercetin-3-O-L-rhamnoside from 366 mg of the crude extract. In our research, we found quercetin-3-β-glucoside in leaf extracts (4.97 μg/mL) and stem extracts (2.01 μg/mL) of *A. negundo*.

9) Isorhamnetin. Isorhamnetin was found in the leaves of *A. glabrum* (Justice *et al.*, 1995). Isorhamnetin-3-O-ruthinoside has also been identified (Backheet, 2003). In our study we identified isorhamnetin in leaf extracts (4.68 μg/mL) and stem extracts (3.31 μg/mL) of *A. negundo*. Glensk *et al.* (2009) identified a triterpene saponin from *A. velutinum* leaf extracts by NMR spectroscopy, 21β-saponin, 22α-O-diangeloylprotoaescigenin, and it exhibited *in vitro* cytotoxic activity against HL-60, B16-F0 and BALB/3T3 cell lines. Also, several authors have analyzed the flavonoid content in maple syrup. Ann (2013) analyzed four grades of maple syrup (extra light, light, medium, and dark) from the 2007 harvest. Twenty-four phenolic compounds were isolated from medium grade syrup and identified by spectral and chemical tests. They were found to have: (a) benzoic acid and several hydroxylated and methoxylated derivatives (gallic acid, 1-O-galloyl-β-d-glucose, and γ-resorcylic acid); (b) cinnamic acid derivatives, coumaric acid, 4-methoxycinnamic acid, caffeic acid, ferulic acid, sinaptic acid, and chlorogenic acid ester); (c) flavonoids, flavanols, catechin and epicatechin, and flavonols of kaempferol and its 3-O-β-d-glucoside, 3-O-β-d-galactoside, quercetin and its 3-O-β-d-glucoside, 3-O-β-L-rhamnoside and 3-O-rhamnoglucoside (rutin). Traces obtained at 280 and 350 nm in the HPLC series of the ethyl acetate soluble fractions of eight samples indicated the presence of several phenolic substances, mostly at very low concentration with some variability in peak heights, but not in retention times, among the syrups.

Authors such as Geoffroy *et al.* (2019) studied hot water extracts of *A. saccharum* bark and shoots, proving that they contain large amounts of phenolic structures that can be used as antioxidant food additives. By performing a replication based on high Performance Liquid Chromatography-High Performance Liquid Chromatography-High Performance Mass Spectrometry (HPLC-DAD-HRMS), it has been showed that hot water extracts of *A. saccharum* bark are rich in simple phenolic compounds and phenylpropanoid derivatives, while the extract of shoots predominantly contains flavonoids, benzoic acids and their complex derivatives, such as condensed and hydrolyzable tannins (Geoffroy *et al.*, 2019).

CONCLUSIONS

Our research revealed that leaf extracts contained a large number of flavonoids compared to stem extracts. *A. negundo* leaf extracts had higher rutin and catechin concentrations; in intermediate concentration were apigenin, gallic acid, ferulic acid, and 2,5-dihydroxybenzoic acid; and in the lowest concentration caffeic acid, quercetin-3-β-glucoside, and isorhamnetin. In *A. negundo* stem extracts there was a higher concentration of ferulic acid, rutin and catechin; in medium concentration were isorhamnetin and quercetin-3-β-glucoside and in the lowest concentration apigenin, but no gallic acid presence, caffeic acid or 2,5-dihydroxybenzoic acid. We report for the first time the presence of gentisic acid in *A. negundo* leaf extracts but not in stem extracts. The method developed to characterize *A. negundo* leaves and stems is rapid and highly sensitive using HPLC. This analytical method can be standardized to serve as a quality analysis for maple

products. With the increasing commercial demand for natural products, phenolic profiles of leaf and stem extracts will help promote these Acer negundo derivatives as new sources of bioactive compounds for the food, nutraceutical, and cosmetic industries.

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