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Phytochemical Study of Leaves of *Muntingia calabura* (Muntingiaceae) from Colombia

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Abstract

Objectives: Contribute to the phytochemical study of the leaves of the plant species *Muntingia calabura* (Muntingiaceae) distributed in Colombia. **Methods:** The leaves of *Muntingia calabura*, collected in the municipality of Honda, Tolima -Colombia (Geographic coordinates: 5 ° 11 ′28′ ′N, 74 ° 44′ 34 ″ W) were extracted by cold maceration with 96% ethanol; this extract was fractionated by liquid /liquid partition and chromatographic methods. The structural elucidation of the isolated compounds was carried out by CG-MS and NMR techniques (experiments 1H, 13C, COSY, J-MOD and HSQC). **Findings:** The phytochemical work developed in *Muntingia calabura* leaves allowed the isolation and identification of a mixture composed of an oxygenated sesquiterpene and a lignan (α-eudesmol and sesamin), hexatriacontane, scopolin and flavonoid 3, 5, 7, 3′, 4′ Pentahidroxiflavona (quercetin) which are identified for the first time for this species.

Keywords: *Muntingia calabura*, *muntingiaceae*, Scopolin, Quercetin, GC-MS

1. Introduction

Muntingia calabura (Muntingiaceae) commonly known as chitato (Colombia) or white capulín (Guatemala, Costa Rica) is a dense shrub of 6 to 20 meters in height, which flowers are white and has multiple edible red fruits. Its leaves are simple, with sizes varying from 3 to 15 cm in width, arranged in two rows per branch, with lanceolate leaf, a toothed edge, with a light green and pubescent shades[1].

Traditionally the whole plant has a medicinal application; an example is the root used as antiseptic and in the treatment before stomach discomfort; flower tea provides a soothing effect on headaches and odontological symptoms as well as on cold. Its fruit has properties such as antitussive and diaphoretic, but its most virtuous property occurs in the treatment of smallpox, measles or urticarial, when rubbing the moistened leaves on the skin[2], evidencing an almost immediate effect.

This plant species are distributed from Mexico through Central America and the Antilles to Colombia, Venezuela, Peru, Brazil and northern Argentina; in Colombia, this plant is located in the coastal plains of the Pacific and Caribbean, in the Magdalena, Cauca, Atrato, Patía, Guaviare, Caquetá and others valleys, as well as in the extensive plains of the Orinoco and the Amazon[3].

From the chemical studies carried out on the species *Muntingia calabura*, flavonoids, chalcones, flavanones, phenolic compounds and steroids are the commonly reported metabolites; in terms of chemotaxonomic importance for this species, flavonoids are the class of chemical compounds that have greater significance for the species, as they are present in most of the organs of this plant, according to studies in countries such as Malaysia, India, Taiwan, Philippines and Indonesia[4]. In Colombia, the species *Muntingia calabura* reports only a study oriented to the preliminary phytochemical analysis fulfilled on fresh leaves, where it was possible to determine the presence of flavonoids, terpenes, phenolic compounds and alkaloids and, in the crust of this

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species, flavonoids, tannins, saponins, terpenes and phenolic compounds[5] were found. The biological activities of these metabolites are directly related to antimicrobial, anti-inflammatory and antioxidant effects[6].

2. Materials and Methods

The plant material was collected in the municipality of Honda, Tolima (Geographic coordinates: 5 ° 11 '28' 'N, 74 ° 44' 34 "W) on October 2015. A control sample was sent to the Colombian National Herbarium for its taxonomic determination which was classified as *Muntingia calabura* ex L. f. under collection number COL 586231. The leaves were separated from fruits, branches and flowers and dried at room temperature, subsequently reduced in size and arranged for the preparation of the extracts.

2.1 Obtaining Extracts and Fractions

Through the cold maceration method, 540 g of dried and reduced size leaves were extracted with hexane. The extraction product was flocculated with acetone in 1:1 proportions, filtered and concentrated under reduced pressure at 40°C.

From the hexane extract obtained (E.HEX.McH), 2.05 g were fractionated by CC using Hexane: AcOEt 8 : 2 mobile faze, obtaining 8 fractions. Fraction 1 (E.HEX.McH.F1) and fraction 5 (E.HEX.McH.F5) were studied. In fraction 1, 103 mg of a white soluble solid in chloroform was precipitated which was denominated compound McH1.

In fraction number 5 (E.HEX.McH.F5), 496 mg of a yellow solid precipitated which was subjected to CC using 7 : 3 CH_2Cl_2 : AcOEt mobile phase obtaining 7 subtractions. In subtraction number 1 (E.HEX.McH.F5.1), 10 mg of a yellow solid were obtained, which was called the McH2 mixture.

The frame, obtained after the extraction with hexane, was extracted with ethanol by the cold maceration method and concentrated under reduced pressure at 40ºC, obtaining the total ethanol extract of leaves of *Muntingia calabura* (E.EtOH.McH). Here, chemical tests of precipitation and/or coloration for the identification of secondary metabolites[7,8] were applied. From this extract, 81.5 g were dissolved in ethanol and flocculated with water in 2 : 1 proportions, and subsequently filtered and subjected to continuous liquid-liquid fractionation with increasing polarity solvents obtaining the fractions of dichloromethane (F.CH_2Cl_2.McH 5, 31 g) and ethyl acetate (F.AcOEt.McH, 3.89 g).

2g of F.CH_2Cl_2.McH were fractionated by CC using a CH_2Cl_2 : AcOEt 8 : 2 mobile phase, obtaining 12 fractions. In fraction 4 (F.CH_2Cl_2.McH.F4), an orange solid (408 mg) precipitated, which was subjected to CCDP separation using as mobile phase CH_2Cl_2 : AcOEt 8 : 2, obtained 3 subtractions. In subtraction number 2 (F.CH_2Cl_2.McH.F4.2, Rf = 0.5), 7.1 mg of a yellow solid called compound McH3 were obtained.

0.7 g of F.AcOEt.McH, were separated by semi-prep liquid chromatography at medium pressure on a Biotage-Isolera. One brand equipment using a 12 g ultra-C-18 snap cartridge using as the mobile phase methanol: water in 4 : 6 proportions with a flow rate of 12ml/min and a gradient up to 100% methanol; obtaining 23 subtractions which were monitored using an ultraviolet detector at 254 nm and 366 nm. In fraction 5, a yellow solid (5.1 mg) was precipitated which was referred to as the McH4 compound.

2.2 Techniques for the Structural Elucidation and Characterization of the Isolated Compounds

For the determination of the mixtures, a chromatograph with SHIMADZU QP2010 plus mass selective detector was used. The separation was performed on a SHRXi-5MS capillary column 30 meters long x 0.25 mm x 0.25 µm with a split mode injection (10 : 1), the entrainment gas used was helium (grade 5.0) with constant flow of 1.2mL/min. The oven temperature setting was: 50°C (2 min) increasing 15°C/min to 200°C (2 min); after, this was increased 10°C/min to 300°C (10 min) for one total analysis time of 34 minutes. The transfer line temperature was 275°C and the ionization chamber was 230°C. The mass spectra were taken on a SHIMADZU MS QP2010 equipped with direct insertion probe and quadrupole mass analyzer. The ionization mode was by Electronic Ionization (EI) at 70 eV and the temperature of the ionization chamber was 230°C. NMR spectra (^1H, ^{13}C, COSY, J-MOD, and HSQC) were taken on a Bruker Advance spectrometer from the NMR laboratory. The analyzes were performed at 300 MHz for ^1H and 75 MHz for ^{13}C, using $CDCl_3$ as solvent and Acetone D_6 with Tetramethylsilane (TMS) as an internal reference.

3. Results and Discussion

3.1 Preliminary Phytochemical Study

The results of the preliminary phytochemical analysis performed on the ethanolic extract of *Muntingia calabura* leaves are listed in Table 1.

The results of the preliminary phytochemical gait (Table 2) show that the plant species *Muntingia calabura* contains certain groups of secondary metabolites such as flavonoids, triterpenes and coumarins.On the contrary, it does not present leucoanthocyanidins, tannins, cardiotonic glycosides, quinones and alkaloids which coincide partially with the only chemical report published in Colombia where a chemical evaluation of the total ethanolic extract of the leaves and fresh bark of *Muntingia calabura* was carried out. The presence of alkaloids, flavonoids, phenolic compounds and terpenes and in the fresh bark showed the presence of tannins, saponins and the absence of alkaloids[6].

Table 1. Results of the preliminary phytochemical analysis

Secondary metabolite tested	Chemical Testing	Result
Flavonoids	Shinoda	+
Leucoantiocyanidines	Rosenheim	-
Tannins	Ferric chloride	-
Triterpenes	Liebermann – Burchard	+
Cardiotonics	Kedde	-
Quinones	Borntranger	-
Alkaloids	Dragendorff	-
Cumarins	Ferric hydroxamate	+

Table 2. Comparison of ^{1}H and ^{13}C NMR chemical shifts of the McH3 compound with those of scopolin[20]

Position	McH3*		Scopolin**	
	RMN ^{1}H	RMN ^{13}C	RMN ^{1}H	RMN ^{13}C
2	-	160,6	-	160,4
3	6,14 (*1H, d, J = 9.4 Hz*)	113,6	6.32 (*1H, d, J = 9.4 Hz*)	113,2
4	7,87 (*1H, d, J = 9.4 Hz*)	131,1	7.96 (*1H, d, J = 9.4 Hz*)	134,1
5	7,18 (*1H, s*)	107,2	7.29 (*1H, s*)	109,3
6	-	146,6	-	146,0
7	-	150,6	-	149,9
8	6,78 (*1H, s*)	102,9	7.16 (*1H, s*)	103,8
9	-	149,5	-	148,9
10	-	112,6	-	112,2
1´	5,22 (*1H, sa*)	100,8	4.95 (*1H, d, J = 7.5 Hz*)	99,7
2´	3,10 – 4,60 (*5H, m, H-2´–H-6´*)	73,8	3,16–4,73 (*5H, m, H-2´–H-6´*)	73,3
3´		77,7		76,7
4´		70,4		69,6
5´		77,8		77,1
6´		61,5		60,6
11	3,90 (*s, 3H*)	55,6	3.82 (*3H, s*)	56,0

* Data obtained at 300 MHz for ^{1}H NMR and 75 MHz in ^{13}C NMR (Solvent: Acetone-D_6)

** Data obtained at 400 MHz for ^{1}H NMR and 100 MHz in ^{13}C NMR (Solvent: DMSO-D_6)[20]

3.2 Isolated Secondary Metabolites

The CC and TLCP separation of the hexane extract and the fractions of dichloromethane and ethyl acetate from *Muntingia calabura* leaves led to the isolation of a hydrocarbon identified as hexatriacontane (McH1), a glycosylated coumarin identified as Escopolin (McH3), a specific flavonoid such as Quercetin (McH4) and a mixture consisting of an oxygenated sesquiterpene and a lignan (α-eudesmol and sesamin) called McH2.

3.3 Compound McH1

The compound McH 1 corresponds to a white solid having a melting point of 74-76°C. This compound was analyzed by GC-MS to obtain a signal with a retention time of 30.688 minutes. The mass spectrum exhibited the base peak at m/z 57; in addition, M-14 losses corresponding to methylene group's characteristic of aliphatic hydrocarbons are observed. Comparison of the mass spectrum with the NIST 08 library shows a 96% coincidence percentage and the comparisons with the data reported in the literature allowed the identification of the compound McH1 as hexatriacontane.

The compound McHl was also analyzed by ^{1}H NMR to confirm its structure. The ^{1}H NMR spectrum showed a triplet at 0.90 ppm (t, J = 7 Hz, 6H) corresponding to two methyl groups. The signal of 1.27 ppm (sa, 68H) indicates the presence of multiple methylene groups. In the low field of the spectrum no type of signal is observed which corroborates that the compound McH1 corresponds to an aliphatic compound. The data obtained in the ^{1}H NMR spectrum, analysis of the mass spectrum and comparison with data reported in the literature confirms that the compound McH1 is hexatriacontane. The hexatriacontane is an aliphatic hydrocarbon used as a marker of digestive transit[9]. It is extracted from different plant species belonging to the families Nyctaginaceae[10], Caesalpiniaceae[11], Caricaceae and Amaranthaceae[12]. There are no reports of this compound for the Muntingiaceae family, which it is reported for the first time for the species *Muntingia calabura*.

3.4 Mixture McH2

The McH2 mixture corresponds to a greenish yellow solid which shows a brown spot on TLC when developed with the vanillin reagent in sulfuric acid. In GC-MS analysis, two major signals were observed in the total ionic current with retention times of 19.525 and 23.508 minutes. Comparison of the mass spectra with those of the NIST 08 library indicates the possible composition of the mixture corresponding to α-eudesmol and sesamin.

In the mass spectrum corresponding to the signal with retention time of 19.525 minutes a signal with m/z = 204 is observed due to the removal of one molecule of water from the molecular ion. In addition, the signals in m/z = 189, 164, and 149 correspond to the base peak of the spectrum. The signal m/z 59 corresponds to the loss of the cyclic part of the molecule which was identified as α-eudesmol.

The α-eudesmol compound is an oxygenated sesquiterpene that has a blocking effect of calcium P/Q channels, being effective in the treatment of cerebral apoplexy and Alzheimer's disease[13]. Different plant species belonging to the Zingiberaceae families[14] and Cupressaceae[15].

As for the mass spectrum corresponding to the signal with retention time of 23.508 minutes, the molecular ion is observed in m/z = 354 and exhibits the signals in m/z = 203, 178, 161, 131 and 149, the latter being the peak spectrum base. After the comparison with the NIST 08 library and the data reported in the literature, it was possible to identify this compound as sesamin.

Sesamin is a neolignan which has been shown to be the major unsaponifiable component of sesame oil (*Sesamum indicum* L), inhibits the absorption and synthesis of cholesterol in rats[16]. Some authors claim that sesamin could be an efficient compound when used as a hypocholesterolemiant[17] has been isolated in several plant families such as Piperaceae[18], Rutaceae[19]. These types of metabolites are identified for the first time for the species *Muntingia calabura*.

3.5 Compound McH3

The compound McH 3 (6.3 mg) was isolated as a yellow, acetone-soluble solid, on TLC was developed with ultraviolet light having fluorescence at 254 nm and 366 nm.

In the ^{1}H NMR spectrum two singlets in δ 7.18 (s, 1H), and δ 6.78 (s, 1H), which due to their chemical shift are attributed to two aromatic protons in para position, these data suggest the presence of a tetra substituted aromatic ring. Signals are also observed for two olefinic protons: one in δ 6.14 (1H, d, J = 9.4 Hz), which by their multiplicity must exhibit couple with an adjacent proton, which is observed at δ 7.87 (1H, d, J = 9.4 Hz).

In the aliphatic zone of the spectrum a singlet is observed which, due to its chemical shift δ 3.90 (s, 3H), can be attributed to the protons of a methoxy group.

The signals of δ 5.22 (sa, 1H) and δ 3.10-4.60 (m, 5H) are indicative that the compound is glycosylated at the C-7 position of a coumarinic nucleus[20].

Analysis of the COSY ^{1}H - ^{1}H spectrum of the McH3 compound allowed to determine the scalar correlation existing between the neighborhood protons; in the spectrum the correlation between the vinyl proton of δ 6.14 (1H, d, J = 9.4 Hz) and the proton of δ 7.87 (1H, d, J = 9.4 Hz) is observed, confirming the presence of two olefinic protons at positions C-3 and C-4 of a coumarinic nucleus.

The HSQC spectrum allowed to establish the connectivity of the protons of the methoxyl group δ 3.90 (s, 3H) with the carbon δ 55,6. For the olefinic protons, the signals of δ 6.14 (1H, d, J = 9.4 Hz) and δ 7.87 (1H, d, J = 9.4 Hz) correlate to a junction respectively with two carbons of a lactone ring of chemical displacement δ 113.6 and δ 131.1. Aromatic protons of 7.18 (s, 1H), and δ 6.78 (s, 1H) show correlation with the carbons of δ 107.2 and δ 102.9 respectively. The other signals observed in the regions of δH 3 - 4.6 and δC 60 - 80 are attributable to the glycosylated part of the molecule.

After analysis of the NMR data and comparison with literature data, it was established that the compound McH3 corresponds to 6-methoxycoumarin-7-O-D-glucoside (scopolin). The spectroscopic data obtained for the compound McH3, as well as the spectroscopic data described for this substance[20], taken at different frequency, ^{1}H-NMR (400MHz), ^{13}C (100MHz) and using DMSO-D_6 as the solvent.

This coumarin has been identified in species of the family Euphorbiaceae[21], Asteraceae[22], Solanácea[23]; for the Muntingiaceae Family this study becomes the first report of this type of substances.

3.6 Compound McH4

The compound McH 4 (10.6 mg) was obtained as a yellow solid with melting point of 315°C, soluble in acetone, which gave positive result to the Shinoda reaction and analyzed by EM. Comparison of the mass spectrum with the NIST 08 library allowed the identification of quercetin with a 97% coincidence. The mass spectrum shows the molecular ion am/ z 302, which is the base peak and also signals in m/z 286, 274, 257, 229, 153, 137, 108 and 69 which according to the literature[24] corresponds to flavonol quercetin. Quercetin is a secondary metabolite widely distributed in different plant families, as well as in the Muntingiaceae family, since this type of metabolite has been reported in studies carried out in Southeast Asia.

4. Conclusion

The present study is a contribution to the phytochemical investigations of the Muntingiaceae family in Colombia and in particular of the genus *Muntingia*, since this is the first report on the constitution of fixed metabolites of a genus that does not have chemical studies in the continent.

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