

Constituents, biological activities and quality control parameters of the crude extract and essential oil from *Arracacia toluensis* var. *multifida*

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Abstract:

Bioassay guided fractionation of an antimycobacterial extract of *Arracacia toluensis* var. *multifida* (Umbelliferae) led to the isolation of isoimperatorin (**1**), osthol (**2**), suberosin (**3**), 8-methoxypsoralen (8-MOP) (**4**), herniarin (**5**), scoparone (**6**), umbelliferone (**7**), dihydroxypeucedanin (**8**), 5-methoxypsoralen (5-MOP) (**9**), isoscopoletin (**10**) and scopoletin (**11**). The isolates were tested against *Mycobacterium tuberculosis* and only **1–4** showed significant activity with MIC values of 64, 32, 16 and 128 µg/mL, respectively. The essential oil showed moderate in vitro antibacterial activity against representative Gram-positive and Gram-negative bacteria. The volatile oil of *Arracacia toluensis* var. *multifida* was analyzed by GC–MS and found to be composed mainly by **2** and **3**. The essential oil ($IC_{50} = 116.4 \pm 23.2$ µg/mL) and the extract ($IC_{50} = 1153.1 \pm 53.2$ µg/mL) of the plant provoked concentration dependent inhibition of the tone and amplitude of the guinea-pig ileum spontaneous contractions; the latter activity was related with the high coumarin content of this species. A suitable (novel and rapid) HPLC method to quantify the major active coumarins of the plant was developed. The method provides also a reproducible fingerprint useful for identity tests of this plant.

Keywords: *Arracacia toluensis* var. *multifida* | Spasmolytic activity | Antimicrobial activity | Coumarins | Antimycobacterial | Umbelliferae

Article:

1. Introduction

Arracacia toluensis var. *multifida* Hemsley (S. Wats.) Mathias & Constance (Umbelliferae) is one of 38 species of the genus *Arracacia* found in the Sierra Madre mountains of Mexico down through the Andes in South America. The plant is a tall perennial herb that grows commonly in

the dry grasslands and oak-juniper woodlands of Mexico ranging in altitude from 2250 to 2950 m over the sea level, from the States of Durango to Hidalgo south through Oaxaca. Local population refer to this species by its Nahuatl name, “acocotli”, or the common Spanish names of “comino rústico”, “hierba del oso” and “neldo”. The fruits and aerial parts of *Arracacia toluensis* var. *multifida* have been used as carminative and digestive stimulant agent along with *Arracacia atropurpurea* (Lehm.) Benth. & Hook (Martínez, 1989). It has also been employed for treating gonorrhoea, fevers and anger (Argueta, 1994). The fruits have been employed in the past as a condiment and the plant eaten as food (Bois, 1904). Although *Arracacia toluensis* var. *multifida* has not been previously investigated from the chemical point of view, studies on the related species *Arracacia vaginata* and *Arracacia nelsonii* led to isolation of several pyranocoumarins, phenylpropanoids and monoterpenoids (Calderon and Ríos, 1975, Delgado and Garduño, 1987). Recently we demonstrated that the crude extract of this species was not toxic for mice or mutagenic when tested by the Lorke and Ames procedures, respectively (Déciga et al., 2007).

Despite the continued use of *Arracacia toluensis* var. *multifida* its composition and pharmacological properties as well as the quality control procedures for the crude drug have not been established yet. Therefore, the present study was undertaken (i) to determine the potential antispasmodic action, chemical composition and active principles of the extract and essential oil of *Arracacia toluensis* var. *multifida* and (ii) to develop an analytical method using HPLC to quantify the most important active principles of the plant. Altogether, the results of these studies will be useful for establishing quality control and preclinical pharmacological parameters for the elaboration of scientific and pharmacopoeic monographs of this Mexican medicinal plant.

2. Materials and methods

2.1. General experimental procedures

IR spectra were obtained on a Perkin-Elmer 599-B spectrophotometer. ^1H NMR (300 and 400 MHz) and ^{13}C NMR (75 and 100 MHz) spectra were recorded in a Varian VXR-300S or Varian Unit Inova spectrometer in CDCl_3 using tetramethylsilane (TMS) as an standard internal. EI mass spectra (ionization energy of 70 eV) were obtained on a HP 5890 spectrometer. HPLC was carried out with a Waters HPLC instrument equipped with Waters Dual 2487 detector. Control of equipment, data acquisition, processing and management of chromatographic information were performed by the Millennium 2000 software program (Waters). The analyses were carried out on a Nova-Pak[®] column (300 mm \times 3.9 mm, 6 μm particle size, Waters). The mobile phase was an isocratic Hex- CH_2Cl_2 -MeOH (80:7:13) system. The flow rate was kept constant at 0.6 mL/min for 30 min. All solvents and reagents were analytical grade *n*-hexane, CH_2Cl_2 , MeOH and EtOAc were obtained from Merck (Darmstadt, Germany) and Burdick & Jackson (Muskegon, USA). GHP[®] membrane filters (0.45 μm) for the mobile phase were supplied by Pall Corporation (New York, USA) and PVDF[®] membranes (0.45 μm) for the preparation of samples before HPLC injection were from Whatman (Germany). GC-MS analysis was carried out in a JEOL JMS-AXOCCHA gas chromatograph interfaced to a Hewlett Packard 5890 mass spectrometer equipped with a 30 m long \times 0.32 mm i.d. \times 0.30 μm film thickness composed of 5% phenylmethylsilicon HP column, connected to an ion trap detector operating in the electron impact mode at 70 eV; carrier gas was He, flow rate 1 mL/min and injection volume

of 20 μL (in CH_2Cl_2). The oven temperature was programmed from 150 to 300 $^\circ\text{C}$ with increase of 10 $^\circ\text{C}/\text{min}$. Column chromatography (CC) was carried out using silica gel 60 (Merck, 70–230 μm ; ASTM, 0.063–0.200 nm). Thin layer chromatography (TLC) was performed on plates with silica gel 60 F₂₅₄ (Merck, 0.25 mm); visualization of plates was carried out using a ceric sulphate (10%) solution in H_2SO_4 .

2.2. Plant material

The aerial parts of *Arracacia toluensis* var. *multifida* were collected southern Mexico City, Mexico on September 1999 (batch 1) and January 2004 (batches 2 and 3). The species was identified by Dr. Robert Bye from the Biology Institute of UNAM. Voucher specimens (Bye & Morales 27040 and Bye 33821, respectively) have been deposited at the National Herbarium of Mexico (MEXU), UNAM, Mexico City.

2.3. Extraction and Isolation

2.3.1. Essential oil

The essential oil (4 g) was obtained by hydrodistillation from the aerial parts (batch 1, 1.0 kg). Major constituents of the essential oil were identified by matching their 70 eV mass spectra with those of the reference library. In the case of **1**, **2**, **4** and **9** the identities were established by comparison with authentic samples.

2.3.2. Extraction and isolation

The air-dried aerial parts (batch 1, 2.0 kg) was ground into powder and extracted by maceration with CH_2Cl_2 –MeOH (1:1, 10 L) at room temperature. After filtration, the extract was evaporated under reduced pressure to yield 160 g of residue.

The organic extract was subjected to open column chromatography on silica gel (1.0 kg) and eluted with a gradient of Hex–EtOAc (1:0 \rightarrow 0:1) and EtOAc–MeOH (1:0 \rightarrow 0:1). Fractions of 200 mL each were collected and then combined according their TLC patterns to yield seven primary fractions (F₁–F₇). Each primary fraction was tested for its antimycobacterial activity. From fraction F₁ (GI of *Mycobacterium tuberculosis* = 99%) eluted with Hex–EtOAc (9:1) crystallized 500 mg (0.025%) of compound **1**. HPLC purification of the mother liquors from fraction F₁ [0.5 mL/min, Hex– CH_2Cl_2 –EtOAc (70:25:5)] yielded **2** (100 mg, 0.005%) and **3** (30 mg, 0.0015%). The retention times were 12.61 and 19.50 min, respectively.

Fraction F₂ (GI of *Mycobacterium tuberculosis* = 100%), eluted with Hex–EtOAc (8:2), was subjected to open column chromatography on silica gel (170 g) and eluted with a gradient of increasing polarity of CH_2Cl_2 –MeOH (1:0 \rightarrow 0:1), afforded five secondary fractions (F_{2-I}–F_{2-V}). Preparative TLC of F_{2-II} using CH_2Cl_2 –MeOH (9:1) yielded **6** (17 mg, 0.00085%, pf 143–145 $^\circ\text{C}$). HPLC purification of F_{2-III} [0.35 mL/min, CH_2Cl_2 –MeOH (99:1)] afforded the known coumarins **1** (50 mg, 0.0025%), **4** (70 mg, 0.0035%) and **5** (10 mg, 0.0005%); retention times: 18.76, 20.48 and 21.94 min, respectively.

Fraction F₄ (GI of *Mycobacterium tuberculosis* = 96%) eluted with Hex–EtOAc (1:1) was rechromatographed on a Si gel (300 g) open column eluting with a gradient CH₂Cl₂–MeOH (1:0 → 0:1). Eight secondary fractions were obtained (F_{4-I}–F_{4-VIII}). Extensive TLC [CH₂Cl₂–MeOH (9:1)] of F_{4-IV} yielded **7** (35 mg, 0.00175%, pf 230–232 °C). Further purification of secondary fraction F_{4-V} by preparative TLC [CH₂Cl₂–MeOH (95:5)] led to isolation of **8** (25 mg, 0.00125%, pf 134–135 °C).

Primary fraction F₅ (GI of *Mycobacterium tuberculosis* = 99%) eluted with Hex–EtOAc (3:7) was rechromatographed on a Si gel column using a gradient system of CH₂Cl₂–MeOH (1:0 → 0:1). Seven secondary fractions were obtained (F_{5-I}–F_{5-VII}). HPLC purification [Hex–CH₂Cl₂–EtOAc (40:30:30)] of F_{5-II} afforded **11** (12 mg, 0.0006%, Rt 9.49, pf 230–232 °C). Finally, preparative TLC of F_{5-VII} using CH₂Cl₂–MeOH (95:5) yielded **9** (20 mg, 0.001%, pf 187–188 °C) and **10** (25 mg, 0.00125%, pf 178–180 °C).

2.4. Quantitative analyses of the crude extract (batches 1–3)

Calibration curves were prepared independently by dissolving the appropriate amount of each compound in CH₂Cl₂ to obtain final concentrations ranging from 0.75 to 25.0 µg/mL. All compounds were detected at 285 nm. A volume of 20 µL was injected. The calibration curves were based on the peak areas of the HPLC chromatograms. The experiments were performed by sextuplicate. The values were expressed in terms of percentage on the basis of the concentration injected. Furthermore, three different concentrations of coumarins **1**, **2**, **4** and **9** were added to a sample of the crude extract. The added concentrations were used to confirm the amount of compounds **1**, **2**, **4** and **9** in the crude extract by means of the standard addition method (ICH Q2(R1), 2005). Validation test materials (coumarins **4** and **9**) were purchased from Sigma (St. Louis, MO, USA) or isolated (**1** and **2**) as described above.

2.5. Antimicrobial disk assay

Gram-positive bacteria [*Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25932)] and Gram-negative bacteria [*Escherichia coli* (ATCC 10536), *Salmonella typhi* (ATCC 9992) and *Pseudomonas aeruginosa* (ATCC 27853)] were used for antimicrobial tests. All microorganisms were provided by Culture Collection at School of Chemistry, UNAM. The bacterial strains were grown in Müeller-Hinton agar (MHA) plates at 37 °C (Mitscher et al., 1987, NCCLS, 1997, NCCLS, 2003, Ojala et al., 2000, Alderman and Smith, 2001). Agar plates containing 1 mL (10⁶ bacteria/mL) of an overnight broth culture were prepared. Disks having a diameter of 6.0 mm were impregnated with 5 µL of each sample at a final concentration of 100, 250, 500, 750 or 1000 µg were placed on the inoculated plates. Similarly, each plate carried a blank disk, with solvent only (DMSO) and antibiotic disk of amikacin (CIC = 0.15 µg/µL; *Staphylococcus aureus*), vancomycin (CIC = 0.10 µg/µL; *Bacillus subtilis*), gentamicin (CIC = 0.20 and 0.05 µg/µL; *Pseudomonas aeruginosa* and *Escherichia coli*, respectively) and ciprofloxacin (CIC = 0.015 µg/µL; *Salmonella typhi*). All the plates were incubated at 37 °C for 24 h. The susceptibility of each microorganism to the samples (crude extract and essential oil) was determined by measuring the sizes of the inhibitory zones on the agar surface around the disks, and values <8 mm were considered as non-active against bacteria. All of the experiments were performed by quadruplicate. The results are reported as critical inhibitory concentration

(CIC) defined as the lowest concentration at which no bacteria growth inhibition was observed after incubation at 37 °C for 24 h.

2.6. Antimycobacterial activity

Activity of the crude extract, fractions and isolated compounds was determined against *Mycobacterium tuberculosis* H₃₇Rv (ATCC 27294) in the Microplate Alamar Blue Assay (MABA) as previously described. The growth of the bacteria is represented as a numerical value called the growth index (GI). The percentage inhibition was defined as $(1 - (\text{GI of test sample}/\text{GI of control}) \times 100)$. The activity of pure compounds was expressed as the lowest drug concentration that affected an inhibition of $\geq 90\%$ relative to untreated cultures (MIC). Rifampin was used as a positive control (MIC = 0.1 µg/mL).

2.7. Spasmolytic activity

2.7.1. Isolated guinea-pig ileum test

All experiments were performed following the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999). Each piece of ileum (1–1.5 cm long) from a freshly killed male guinea pig was suspended in a tissue chamber containing 10 mL of Tyrode solution with composition (mM): NaCl 149.2; KCl 2.7; MgCl₂·6H₂O 2.1; CaCl₂ 3.6; NaH₂PO₄ 0.4; NaHCO₃ 11.9; glucose 5.0; pH 7.4, at 37 °C, continuously gassed with carbogen (95% O₂ + 5% CO₂). The contractions were recorded with an isometrical force transducer (Grass FT 03) connected to a MP100 Manager Biopac System polygraph. The data were digitalized and analyzed by mean of software for data acquisition (Acknowledge 3.7.3). Tissues were placed under a resting tension of 1.0 g and allowed to stabilize for 45 min and they were washed with fresh Tyrode solution at 15 min intervals before starting the experiments. After the stabilization period the tissues were contracted with carbachol (1×10^{-6} M) to assess its viability. The extract and essential oil suspended in 0.5% Tween 80 in water were added to the bath in a volume of 100 µL at final concentrations of 3, 10, 30, 100, 300 and 1000 µg/mL. All responses were recorded during 10 min. The antispasmodic activity of the extract was assessed by its ability to prevent the contractions induced by a submaximal concentration experimentally determined of acetylcholine (1×10^{-4} M), histamine (1×10^{-3} M), serotonin (1×10^{-4} M) and BaCl₂ (1×10^{-2} M). Papaverine was used as positive control. All the results are expressed as the mean of six experiments \pm S.E.M. Concentration–response curves (CRC's) for the extract and oil were plotted and the experimental data were adjusted by nonlinear least squares, curve fitting program (SigmaStat). The statistical significance ($p < 0.05$) of differences between means was assessed by an analysis of variance (ANOVA) followed by a Turkey's test.

3. Results and discussion

3.1. Antibacterial activity

Since *Arracacia toluensis* var. *multifida* is used as an antiinfective agent, a CH₂Cl₂–MeOH (1:1) extract was tested against *Mycobacterium tuberculosis* using the Alamar Blue procedure (Collins and Franzblau, 1997). The results revealed that the extract significantly inhibited

Mycobacterium tuberculosis growth (GI = 99%). Bioassay guided fractionation of the active extract using silica gel column chromatographic revealed that primary chromatographic fractions F₁–F₅ concentrated the activity (see Section 2.1). Extensive chromatographic separation of the active fractions led to the isolation of 11 coumarins (Fig. 1) which were identified by IR, NMR and MS methods as isoimperatorin (**1**), osthol (**2**), suberosin (**3**), 8-methoxypsoralen (8-MOP) (**4**), herniarin (**5**), scoparone (**6**), umbelliferone (**7**), dihydroxypeucedanin (**8**), 5-methoxypsoralen (5-MOP) (**9**), isoscopoletin (**10**) and scopoletin (**11**) (Ivie, 1978, Harkar et al., 1984, Estevez-Braun and González, 2002).

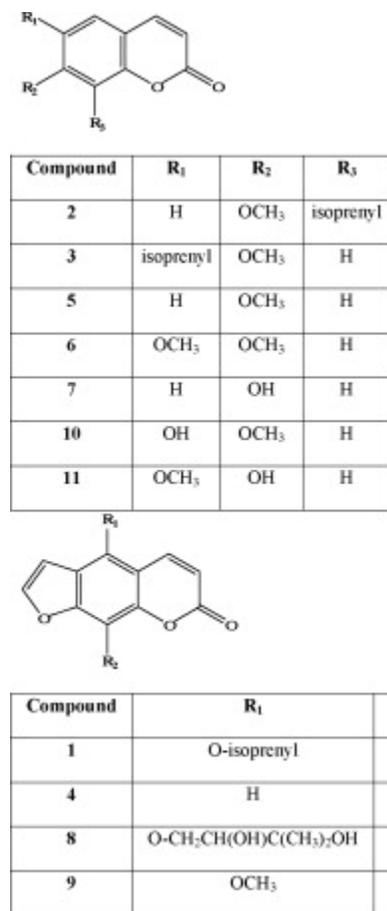


Fig. 1. Structures of coumarins **1**–**11**.

In general the spectroscopic data were in agreement with those previously described for compounds **1**–**11** (Ivie, 1978, Harkar et al., 1984, Estevez-Braun and González, 2002). Briefly, the common feature of all structures is the benzo- α -pyrone unit differing only in the nature of the substituents (i.e. hydroxy, methoxy, isoprenyloxy, isoprenyl or furan) along the benzene core. The NMR data of compounds **1**–**11** showed strong similarities albeit partial. The ¹³C NMR and IR spectra confirmed the α -pyrone unit since the characteristic carbonyl absorption was observed at ~ 160 ppm and ~ 1720 cm⁻¹, respectively. In all cases, the ¹H NMR spectra in the region between 6 and 8.2 ppm showed the typical AB system for H-3 and H-4 and signals for the aromatic. The nature of the substituents was determined by means of the analysis of the NMR spectra which revealed resonances for methoxyl, hydroxyl, furan or different isoprenyl moieties. The position of the substituents in the aromatic ring was established on the basis of the splitting

pattern of the aromatic hydrogens (Ivie, 1978, Harkar et al., 1984, Estevez-Braun and González, 2002).

The isolates were tested against *Mycobacterium tuberculosis* and only compounds **1–4** showed significant anti-TBC activity with MIC values of 64, 32, 16 and 128 µg/mL, respectively. Compounds **5–11** displayed MIC values higher than 128 µg/mL. The better activity of **1–3** over the remaining compounds are in agreement with the observations of Schinkovitz et al. (2003) who found that the presence of an isoprenyl unit attached to the carbocyclic ring of some coumarins provoked an increment of their antimycobacterial properties. Also these authors demonstrated that umbelliferone (**7**) was weakly active against *Mycobacterium fortuitum*.

The extract and essential oil of *Arracacia toluensis* var. *multifida* was also tested against an appropriated battery of Gram-positive and Gram-negative bacteria using the disk diffusion method. The crude extract was active against *Staphylococcus aureus* and *Bacillus subtilis*; the CIC values were 9.92 and 27.7 µg/µL, respectively. On the other hand, the essential oil showed moderate in vitro antibacterial activity against both Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*) with CIC values of 50.4, 99.8, 150.2, 150.1 and 147.9 µg/µL, respectively. The susceptibility of common Gram-negative and Gram-positive to the tested material was poorer in comparison with that of standard antibiotics.

3.2. Essential oil composition

The volatile oil of *Arracacia toluensis* var. *multifida* was analyzed by GC–MS and found to be composed mainly by osthol (**2**), suberosin (**3**), 8-MOP (**4**), 5-MOP (**9**), benzyl alcohol, terpinen-4-ol and α -cadinene. Near a 50% of the constituents were identified, being compounds **2** and **3** the major active principles. In the case of compounds **2–4** and **9** the identities were established by comparison with authentic samples. The Rt's (retention times) for compounds benzyl alcohol, terpinen-4-ol, α -cadinene, **9**, **4**, **2** and **3**, were 9.13, 11.66, 16.24, 23.27, 23.46, 24.18 and 24.84 min, respectively.

3.3. Spasmolytic activity

The use of *Arracacia toluensis* var. *multifida* as carminative and digestive stimulant agent suggested that this species could have spasmolytic properties. In order to corroborate this hypothesis, the effect of both a CH₂Cl₂–MeOH (1:1) extract and the essential oil prepared from the aerial parts of the plant on the spontaneous contraction of the guinea-pig ileum was assessed. The results of the pharmacological testing revealed that both preparations provoked a concentration dependent inhibition of the tone and amplitude of the guinea-pig ileum spontaneous contractions (Fig. 2a). The essential oil (IC₅₀ = 116.4 ± 23.2 µg/mL) was more active than the crude extract (IC₅₀ = 1153.1 ± 53.2 µg/mL), although three times less active than papaverine (IC₅₀ = 35.0 ± 1.4 µg/mL) used as positive control (Fig. 3). The influence of the extract and essential oil on histamine-, acetylcholine-, serotonin- and BaCl₂-induced smooth muscle contractions was also investigated (Fig. 2b and c). The crude extract as well as the essential oil antagonized the contractions exerted by acetylcholine (1 × 10⁻⁴ M), histamine (1 × 10⁻³ M), serotonin (1 × 10⁻⁴ M) and BaCl₂ (1 × 10⁻² M) as is showed resumed in Table 1.

Altogether this information revealed that the smooth muscle relaxant activity displayed by the extract and essential oil of *Arracacia toluensis* var. *multifida* involved an unspecific inhibition of the calcium influx into the smooth muscle cells (Rojas et al., 1995, Rojas et al., 1996).

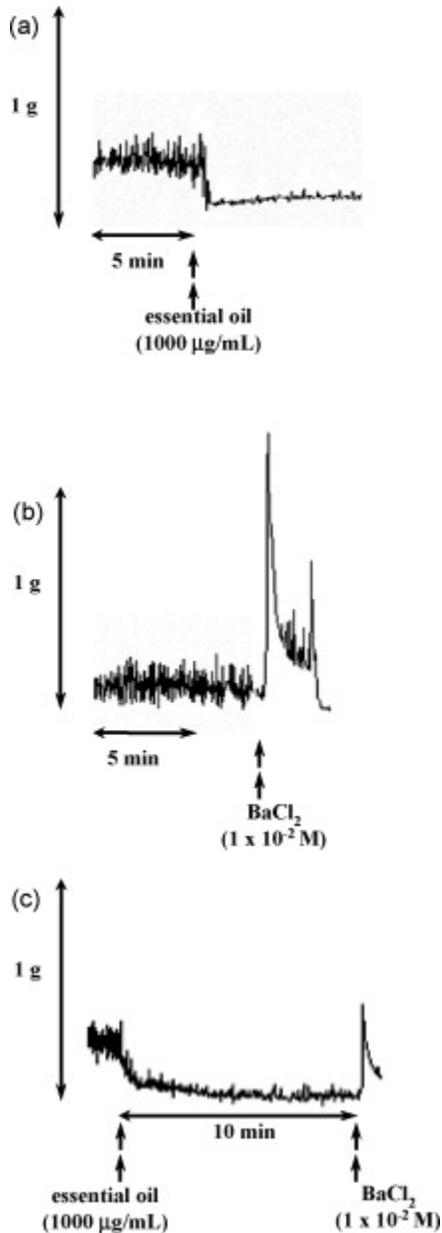


Fig. 2. Tracing of guinea-pig ileum muscle showing the response contractile by (a) essential oil (1000 µg/mL), (b) spasmogen (BaCl₂ 1 × 10⁻² M) and (c) spasmogen after treatment with essential oil (1000 µg/mL) during 10 min. The arrows indicate the treatment added to the bathing fluid.

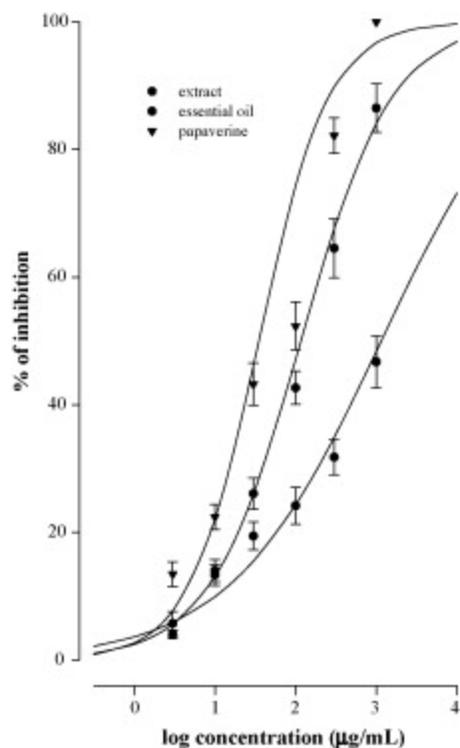


Fig. 3. Concentration–response curves showing the relaxatory effect of the extract and essential oil from *Arracacia toluensis* var. *multifida* on the spontaneous contractions of isolated guinea-pig ileum. Values are expressed as the percentages of inhibition of contractile responses calculated as the mean from six different animals \pm S.E.M., $p < 0.05$.

Table 1. Effect of extract and essential oil from *Arracacia toluensis* var. *multifida* on the contractions induced by acetylcholine, histamine, serotonin and BaCl₂ on isolated guinea-pig ileum

Sample	Acetylcholine (1×10^{-4} M)	Histamine (1×10^{-3} M)	Serotonin (1×10^{-4} M)	BaCl ₂ (1×10^{-2} M)
IC ₅₀ (μ g/mL) \pm S.E.M.				
Extract	38.7 \pm 12.3	53.4 \pm 32.5	15.2 \pm 5.1	27.8 \pm 5.0
Essential oil	30.0 \pm 2.8	23.7 \pm 5.0	11.1 \pm 1.5	15.4 \pm 4.9
Papaverine ^a	12.7 \pm 4.1	12.8 \pm 4.3	9.0 \pm 1.8	11.3 \pm 3.2
E_{\max} (10^{-3} μ g/mL) \pm S.E.M.				
Extract	97.2* \pm 1.1	98.2* \pm 0.7	99.7* \pm 0.1	99.3* \pm 0.5
Essential oil	98.8* \pm 0.6	98.4* \pm 0.4	98.8* \pm 0.1	98.9* \pm 0.5
Papaverine ^a	100.0* \pm 0.0	100.0* \pm 0.0	100.0* \pm 0.0	100.0* \pm 0.0

Values are expressed as the percentages of inhibition of contractile responses calculated as the mean from six different animals \pm S.E.M., * $p < 0.05$ (ANOVA).

^a Positive control.

The presence of compounds **2** (Li et al., 1994, Chen et al., 2000, Che-Ming et al., 2004), **4**, **9** (Call and Green, 1956, Novak et al., 1967), **6** (Liu et al., 2002) and **10** (Reisch et al., 1966) with known spasmolytic properties could account for the antispasmodic activity of the extract and oil of *Arracacia toluensis* var. *multifida*. Osthol (**2**) and 5-MOP (**9**) antagonized the contractions evoked by acetylcholine, histamine, BaCl₂ and KCl on isolated rat ileum preparations. In addition, Teng et al. (1994) reported that osthol (**2**) inhibited calmodulin sensitive phosphodiesterase (PDE1) on trachea smooth muscle.

3.4. Quantitative HPLC analysis

To develop a suitable HPLC fingerprint and a method to quantify the major coumarins of the extract of *Arracacia toluensis* var. *multifida*, three different samples were collected in two different seasons (Fig. 4). The calibration curves (Table 2) indicated the linearity of the detector response for the four standard analytes from 0.75 to 25.0 µg/mL. The limit of detection (LOD) ranged from 0.15 to 0.47 µg/mL for the standard analytes. The reproducibility and repeatability of the analytical method were evaluated in terms of the intermediate precision by analyzing three replicates of six samples of stock solution (12.5 µg/µL) on 3 different days. The relative standard deviation (R.S.D.) [$n = 6$] and coefficient of variation (CV) were calculated for each batch. The results indicated that their chromatographic pattern were similar showing in each case the presence of four peaks assigned as **1**, **2**, **9** and **4**. The CV values (≤ 3.0) and the precision of retention times and peak areas of compounds **1**, **2**, **4** and **9** for replicated injections. In order to determine the accuracy of the method, one sample was spiked with a known amount of the standard analytes **1**, **2**, **4** and **9**. Recovery ranges were found to be between 97.5 and 102.5%. Finally, this HPLC method was used to quantify the coumarins **1**, **2**, **4** and **9** in the crude extract of *Arracacia toluensis* var. *multifida* (Table 3).

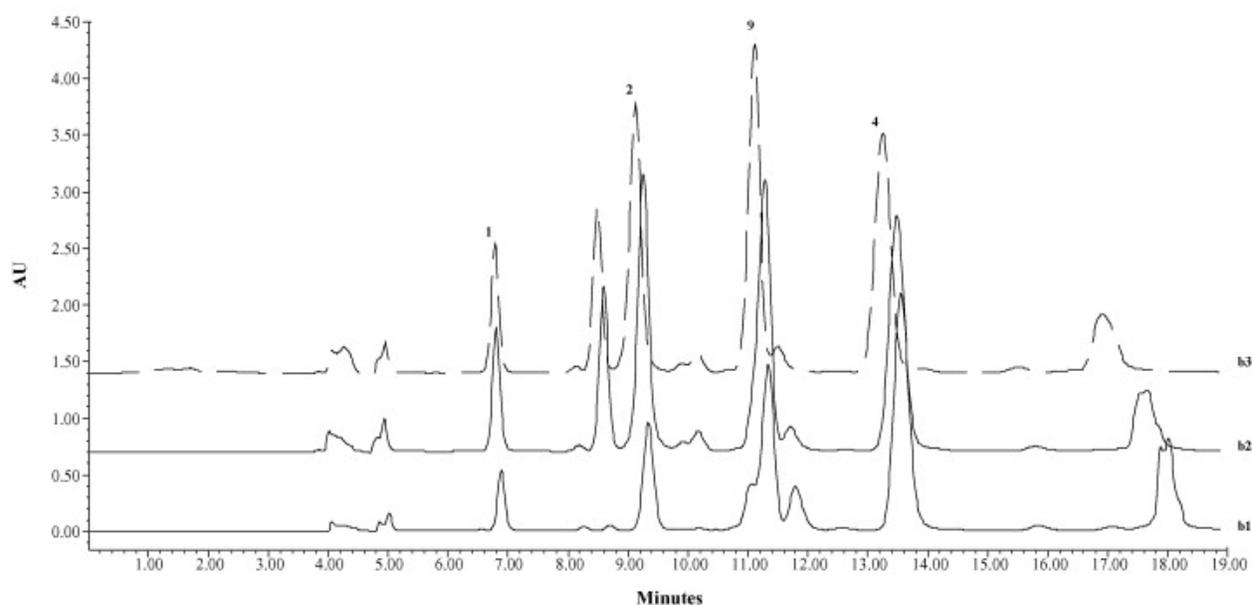


Fig. 4. HPLC chromatogram of crude extract from *Arracacia toluensis* var. *multifida* of three different batches (**b1–b3**).

Table 2. Calibration data [regression equation and correlation coefficient (r^2)] and LOD for coumarins **1**, **2**, **4** and **9**

Analyte	Regression equation	r^2	LOD ($\mu\text{g/mL}$)
1	$y = 1.34 \times 10^8x + 4.14 \times 10^4$	0.9999	0.47
2	$y = 1.10 \times 10^8x + 1.26 \times 10^5$	0.9999	0.19
4	$y = 1.65 \times 10^8x + 6.58 \times 10^5$	0.9989	0.15
9	$y = 1.53 \times 10^8x - 2.45 \times 10^5$	0.9999	0.22

Table 3. Quantification of the coumarins **1**, **2**, **4** and **9** (mg/g of extract) by HPLC analysis

Compound	Batch 1	Batch 2	Batch 3
1	9.8 ± 1.0	16.6 ± 0.8	15.8 ± 1.2
2	25.8 ± 4.0	60.2 ± 5.8	57.0 ± 6.4
4	67.4 ± 1.8	61.8 ± 3.8	58.8 ± 3.0
9	46.0 ± 5.8	42.0 ± 1.0	39.4 ± 1.4

4. Conclusions

The organic extract and the essential oil from the aerial parts of *Arracacia toluensis* var. *multifida* showed significant spasmolytic activity; this action could be useful to regulate gastrointestinal disorders associated with indigestion or anomalous gastrointestinal motility. The crude extract also showed noted antimycobacterial action against *Mycobacterium tuberculosis*; the latter effect could be related with the use of the plant as an anti-infective agent. The biological properties for *Arracacia toluensis* found in this study were related with its high coumarin content. Four of the coumarins isolated and characterized were active either as spasmolytic or antimycobacterial agents, therefore, it is highly probable that these compounds contributed synergistically to the pharmacological actions exhibited by the crude extract or essential oil of *Arracacia toluensis* var. *multifida*. The antimycobacterial activity of isoimperatorin (**1**), osthol (**2**), suberosin (**3**) and 8-methoxypsoralen (8-MOP) (**4**) is newly described.

The coumarins isolated from *Arracacia toluensis* var. *multifida* has not been isolated from the related species so far investigated which contain pyranocoumarins, phenylpropanoids and monoterpenoids.

A suitable (novel and rapid) HPLC method to quantify the major active coumarins of the plant was developed. The method provides also a reproducible fingerprint useful for identity tests of this plant.

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