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HPLC quantification of two isomeric triterpenic acids isolated from *Mitracarpus scaber* and antimicrobial activity on *Dermatophilus congolensis*

Fernand Gbaguidi [a,b], Georges Accrombessi [b], Mansourou Moudachirou [b], Joëlle Quetin-Leclercq [a,*]

[a] *Laboratoire de Pharmacognosie, Unité CHAM, Département de Pharmacie, Université Catholique de Louvain, Av. E. Mounier 72-30, B-1200 Bruxelles, Belgium*
[b] *Laboratoire de Pharmacognosie et Huiles Essentielles, Faculté des Sciences de la Santé, École de Pharmacie, Université d' Abomey Calavi, 01 BP 188 Cotonou, Bénin*

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Abstract

Oleanolic (OA) and ursolic acids (UA) were isolated for the first time from the alcoholic extract of *Mitracarpus scaber* possessing antimicrobial effects on *Dermatophilus congolensis*. These two triterpenic acids were also active (MIC 15 μg/ml) on this causative agent of dermatophilosis in African animals.

To quantify OA and UA in *M. scaber*, a new, simple and rapid high-performance liquid chromatography (HPLC) method compatible with MS detection was developed and validated. The mobile phase acetonitrile:H_2O (85:15, v/v) was pumped through a C18 octadecylsilyl silica column at a flow rate of 0.6 ml/min and the eluate was monitored at 215 nm. The calibration curves constructed between 0.5 and 10 μg/ml showed linear relationships with good R^2 values. The developed method was precise and reproducible with relative standard deviations (RSD) for these two active constituents between 0.22–2.06% (intraday) and 1.61–3.72% (interday) for concentrations from 0.5 to 6 μg/ml. Limits of detection and quantification were, respectively, 0.2 and 0.5 μg/ml.

Keywords: HPLC; *Mitracarpus scaber*; *Dermatophilus congolensis*; Triterpenic acids

1. Introduction

Dermatophilosis is an enzootic bacterial skin infection of bovines in tropical and subtropical countries [1]. It is caused by a gram positive bacterium, *Dermatophilus congolensis* [2]. Dermatophilosis infection in cattle may lead to death of the animal, and cause economic losses to farmers, in the case of a severe acute form.

Previous works have evaluated the antibacterial activity of aqueous and alcoholic extracts of the aerial parts of *Senna alata*, *Lantana camara* and *Mitracarpus scaber* in vitro on *Dermatophilus congolensis*, and have shown that alcoholic extracts of *Mitracarpus scaber* were the most active [3]. Furthermore, ointments containing an alcoholic extract of *M. scaber* used topically showed the higher efficiency against bovine dermatophilosis and cured tested animals without recurrence [4].

M. scaber Zucc. ex Schult + Scult.f. (RUBIACEAE), a traditional African medicinal herb, has been used to treat various human skin diseases including eczema [5] and ringworm [6]. It has been shown to contain azaanthraquinone [7], psoralen, Kampferol-3-*o*-rutinoside, gallic acid, 3,4,5-trimethoxy benzoic acid, 4-methoxyacethophenone and 3,4,5-trimethoxyacetophenone [8]. These four last compounds had no antimicrobial activity on *D. congolensis* in our experiments.

* Corresponding author. Tel.: +32 2 7647254; fax: +32 2 7647253.
E-mail address: Leclercq@cham.ucl.ac.be (J. Quetin-Leclercq).

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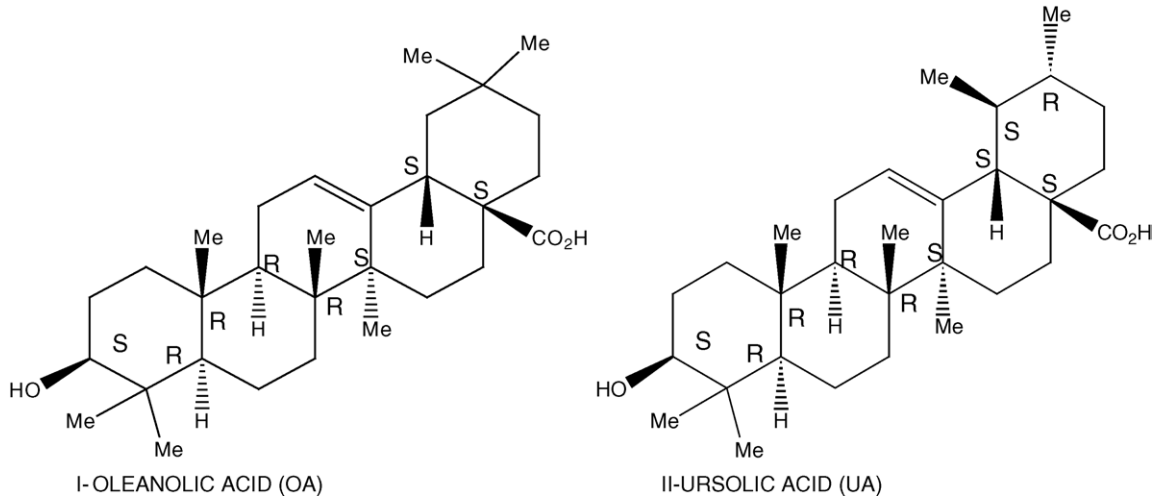

Fig. 1. Chemical structures of oleanolic acid and ursolic acid.

From the alcoholic extract, we identified for the first time by activity guided fractionation two triterpenic acids: oleanolic acid (OA) and ursolic acid (UA) (Fig. 1), by comparison of their chromatographic (TLC) behaviours with reference compounds before and after derivatisation [9], by LC–MS (APCI positive ions analysis) [10–11] and NMR [12] after purification by medium-pressure liquid chromatography (MPLC) [13] on silica gel.

Both OA and UA were reported to have biological activity including anti-inflammatory [14–16], antiprotozoal [17] and antimicrobial (against some gram positive bacteria) properties [18–21] and cytotoxicity to cancer cells [22]. OA also possesses hepatoprotective [23], anti-ulcer activities [24], and UA exerts anti-tumor activity through enhancing the production of both nitric oxide and tumor necrosis factor −α via nuclear factor-kappa β activation in the resting macrophage [25]. As *M. scaber* extracts have antimicrobial activity against *D. congolensis*, we analysed the effects of these two acids on *D. congolensis*.

In order to obtain quantitative information on the amounts of these two molecules in the plant, we tested several methods described in the literature [26–30] but none of them suited to our extracts as OA and UA peaks were not well separated from other constituents. Furthermore, the method recently published by Chen et al. was not compatible with MS detection [31]. So we developed a new high-performance liquid chromatography (HPLC/UV) method compatible with MS detection to quantify these compounds in the plant aerial parts and validated it.

2. Experimental

2.1. Plant material and crude sample preparation

The aerial parts of the plant were collected in the area of Cotonou, Abomey-Calavi and were identified and authenticated by the National Herbarium of the University of Abomey-Calavi in the Republic of Benin, where a voucher specimen was deposited (nr: AA.6272/HNB). The plant was first dried at room temperature for 5 days during which it was turned over every day, then dried in an oven at 50 °C for 48 h and subsequently reduced to coarse powder [32] using a grinder and stored at room temperature. Of this powder, 500 g were macerated in 4 l of ethanol (Merck) for 72 h by constant shaking. This extract was filtered and concentrated by vacuum evaporation under reduced pressure.

2.2. Purification

The alcoholic extract was fractioned by column chromatography (silicagel 0.062–0.2 mm, column: 2.2 cm × 20 cm) using a gradient of solvents (Table 1). Fractions were monitored by TLC and assembled in three fractions (Table 1). Two grams of the alcoholic extract or F1 were dissolved in 50 ml of 1N NaOH and extracted 3 times with 50 ml hexane. The aqueous phase was acidified with 1N H_2SO_4 and extracted three times with 50 ml of dichloromethane. After evaporation of the dichloromethane, the residue was dissolved in 5 ml of toluene and fractionated

Table 1
Column fractions

Column fractions	Solvents (150 ml)	Assembled fractions
1	Petroleum ether	F1
2	Petroleum ether–toluene (1:1)	
3	Dichloromethane	
4	Dichloromethane–ethyl acetate (1:1)	
5	Ethyl acetate	F2
6	Dichloromethane–acetone (1:1)	
7	Acetone	F3
8	Acetone–methanol (1:1)	
9	Methanol	

by MPLC on silica gel (Lichroprep Si 60 Merck, omnifit glass column OM 6427 15 mm × 750 mm, with Gilson SC-Type Pump) by elution with successively 140 ml of toluene, 120 ml of a toluene–ethyl acetate mixture (90:10, v/v) and 600 ml of toluene–ethyl acetate–methanol mixture (80:18:2, v/v/v). The last eluate was collected (by a fraction collector) in 6 ml fractions. Fractions 40–65 were evaporated and concentrated.

2.3. Antimicrobial tests

The agar dilution method (National Committee for Clinical Laboratory Standards, 1990) was used to determine the Minimal Inhibitory Concentration (MIC: the minimal concentration completely inhibiting the growth of the micro-organism) of *M. scaber* extracts and tetracycline as positive control (Sigma). The micro-organisms were grown overnight on Tryptone Soya broth (Oxoid Ltd., England). Inoculates of 10^3–10^4 CFU were spotted with a multipoint inoculator A400 (Denley Instruments Ltd., England) on Muller-Hinton agar supplemented with the extract or antibiotic at concentrations ranging from 1000 to 2 μg/ml for the extract and from 64 to 0.5 μg/ml for the antibiotic and the two acids. Blank (DMSO used to dissolve extracts) was included. The plates were incubated for 4 days at 37 °C. Tests were performed at least in duplicate.

2.4. HPLC and LC–MS analysis

The HPLC system consisted of a Merck-Hitachi AS-2000A autosampler equipped with a 7725i Rheodyne injector with a 20 μl loop (Cotati, CA, USA), a Kontron 320-model pump (Milan, Italy) and a Merck-Hitachi L4000 UV detector. Chromatographic separations were performed on a SPHERISORB® ODC column, 25 cm × 4.6 mm, 5 μm particle size. The temperature of the column was kept constant at 25 °C by using a Merck-Hitachi T6300 column heater. The mobile phase (acetonitrile–H_2O, 85:15, v/v) was delivered to the column at a flow rate of 0.6 ml/min and the eluate was monitored at 215 nm. Chromatograms were processed by using a Borwin version 1.50 integration program running on Windows NT 4.00.

The LC–MS system was equipped with a Merck-Hitachi L6200 pump, a manual 7725i, Rheodyne injector and LCQ advantage S/NLAD00293 detector. The chromatographic separations were performed on a SPHERISORB® ODC column, 25 cm × 4.6 mm, 5 μm particle size. Mass spectra were acquired using an LCQ mass spectrometer, equipped with an APCI source. Data acquisition and processing were performed with Xcalibur software. All solvent used were of HPLC grade.

2.4.1. Calibration curve

A series of OA and UA (Sigma) standards were prepared by diluting a stock solution of OA or UA (10 mg/100 ml mobile phase) with mobile phase to obtain 0.5, 1, 2, 3, 4, 6 and 10 μg/ml. The stability of these standards was investigated at room temperature.

2.4.2. Preparation of sample solutions

Twenty milliliters of dichloromethane were added to 2 g of plant material (obtained as described in Section 2.1) and weighted. Plant was then extracted during 90 min by sonication and regular stirring with a glass rod (every 30 min). After readjusting the weight by adding dichloromethane, 1 ml of supernatant was transferred to a clean tube and dried by a stream of nitrogen. The residue was then dissolved in 3 ml of mobile phase and filtered through a hydrophobic PTFE FLUOROPOR® filter. Twenty microliters of this sample solution were injected into the HPLC system.

2.4.3. Recovery

To 2 g of plant powder, obtained as described in Section 2.1, 3 μg (1 ml of 3 μg/ml solution in the mobile phase) of OA and UA were added and dried, then the mixture was extracted as described in Section 2.4.2. The same extraction procedure was used on 2 g of the powder without adding any standards. Aliquots of both extracts were analysed by HPLC and the recovery was calculated as follows:

$$\text{Recovery}\,(\%) = \left[\frac{A - B}{C}\right] \times 100$$

where A is the quantity of OA and UA in the spiked powder, B is the quantity of OA and UA in the powder without added standards and C is the quantity of added OA and UA.

2.4.4. Repeatability and precision

Precisions were determined by analysing the 0.5, 3 and 6 μg/ml standards several times ($n = 5$) during the same day or on various ($n = 5$) days by two different operators.

3. Results and discussion

Fractionation by column chromatography of the alcoholic extract allowed us to obtain three fractions (Table 1) which were tested for antimicrobial activity on *D. congolensis*. The most active fraction (F1) contained compounds with the same characteristics as triterpenic acids by TLC on silica gel 60 F_{254} Merck with a mixture of toluene–ethyl acetate–methanol (80:18:2, v/v/v) as mobile phase and revealed by anisaldehyde reagent. This fraction was partitioned by liquid–liquid separation and purified by MPLC. The presence of OA and UA was confirmed by the Brome test [9], by HPLC analysis before and after spiking the extracts with pure OA or UA, HPLC/MS [10–11] and NMR [12].

Table 2 shows the activity of the extract, fractions and both triterpenic acids on *D. congolensis* compared with tetracycline. The results show that isolated acids though less active than tetracycline, possess an activity at relatively low concentration (MIC: 15 μg/ml).

Table 2
MIC values against *D. congolensis* for OA and UA, fractions and the alcoholic extract of *Mitracarpus scaber*

Blank with 500 μl of DMSO	Tetracyclin	Oleanolic acid	Ursolic acid	Alcoholic extract	F1	F2	F3
NA	1 μg/ml	15 μg/ml	15 μg/ml	1000 μg/ml	125 μg/ml	1000 μg/ml	NA

NA: no activity.

A HPLC/UV method compatible with MS detection was subsequently developed to quantify oleanolic acid and ursolic acid in the plant. Typical HPLC/UV and HPLC/MS chromatograms are shown in Fig. 2 illustrating the separation of OA and UA in an extract of *M. scaber*. During the development of the method it was shown that both compounds were stable in stock solutions prepared with the mobile phase for at least 3 months. A detection wavelength of 215 nm was chosen because both OA and UA showed an optimal absorbance at that wavelength in the selected mobile phase. The detection limit (taken as a peak height 3 times the noise level) was determined as 0.2 μg/ml and the quantification limit (taken as a peak height 10 times the noise level) was calculated as 0.5 μg/ml for the two acids.

For both phytochemicals calibration curves were constructed in the range of 0.5–10 μg/ml. The method showed a linear relationship between peak areas and known concentrations of OA and UA between 0.5 and 10 μg/ml. Analysis of these standards on 5 different days yielded the following mean calibration curves for OA and UA, respectively: $y = 45.4x - 2.2$ (R^2 was between 0.98 and 0.99) and $y = 38.2x - 6.3$ (R^2 was between 0.97–0.99). The inter- and intra-day variations for the determinations of OA and UA were less than 4% at concentrations of 0.5, 3 and

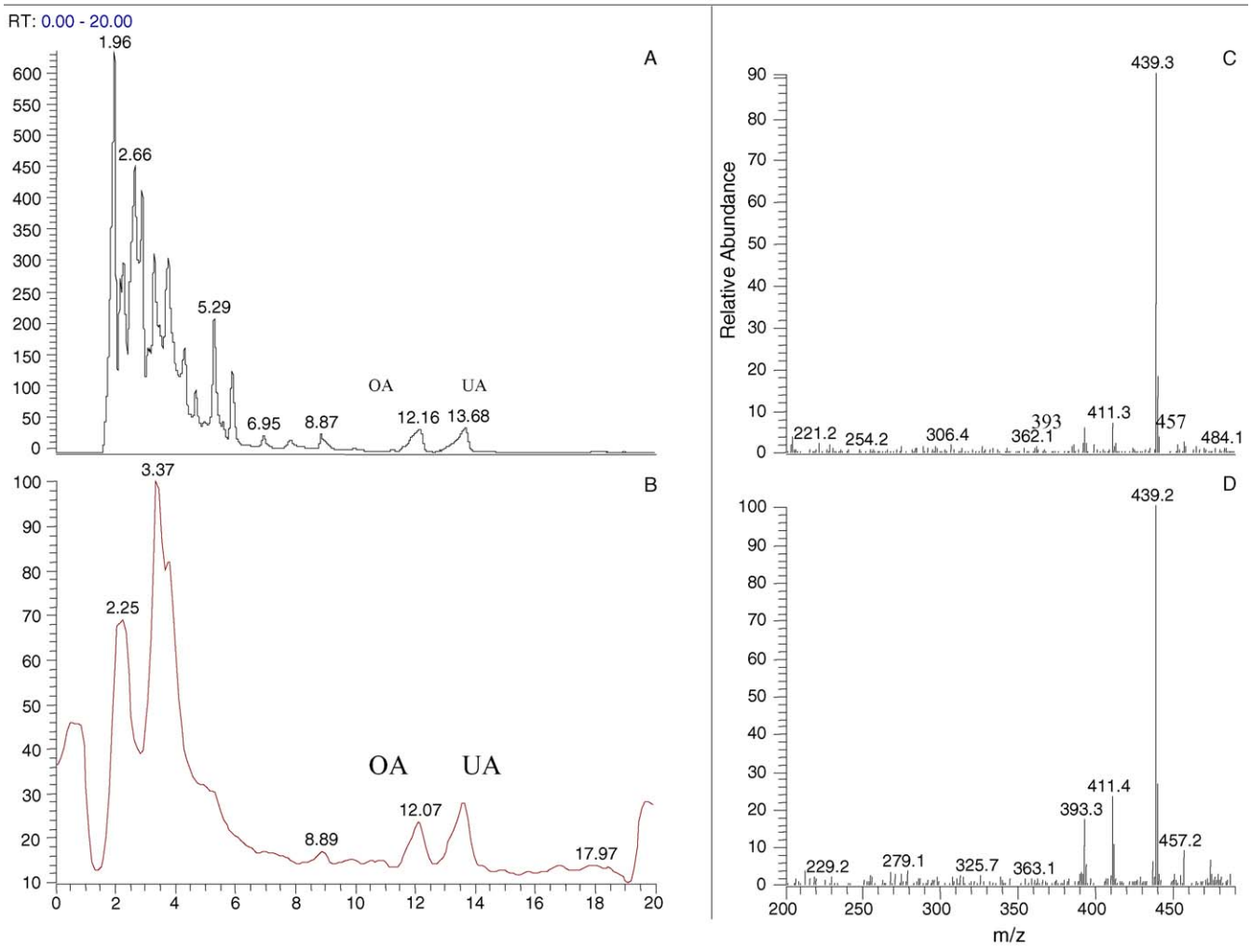

Fig. 2. LC/UV (215 nm: A) and LC/MS (TIC: B) chromatograms of the plant dichloromethane extract and full MS spectra (*m*/*z* 200–490) of OA (C) and UA (D) peaks.

Table 3
Inter- and intra-day precision for the quantification method of OA and UA in standard solutions

Standard	Concentration (μg/ml)	RSD	
		Intraday ($n = 5$)	Interday ($n = 5$)
OA	0.5	2.06	2.52
	3	0.22	3.72
	6	0.22	2.13
UA	0.5	1.79	3.39
	3	0.98	1.79
	6	2.13	1.61

6 μg/ml (Table 3). Recoveries of OA and UA were 97.4 and 98.2%, respectively, with relative standard deviations of 1.49 and 0.98% ($n = 5$).

Peaks in the chromatograms obtained from plant samples were identified by comparison of their retention times with those of authentic standards (OA and UA) and by LC–MS which was also used to confirm the peak purity (Figs. 2 and 3).

This new HPLC/UV method was used to quantify OA and UA in *M. scaber* extracted as described in Section 2.4.2. By taking the correction factors into account the quantities of OA and UA in 2 g of plant material were estimated to be 86 ± 0.94 and 250 ± 15.62 μg, respectively, for the dichloromethane extraction procedure. This corresponds to 0.0043% of OA and 0.0125% of UA in the plant material sample. As the yield of the alcoholic extract was 15.9%, it contains maximum 0.027 and 0.0786% of UA and OA, corresponding to a total of 1.056 μg of these two acids in 1000 μg of extract. This implies that OA and UA may explain a part of the observed effect of this alcoholic extract on *D. congolensis* in vitro.

In conclusion, we developed a new HPLC/UV method for the quantification of oleanolic acid and ursolic acid in *M. scaber* extracts and validated it. This method was fully compatible with MS detection and allowed us to quantify these two antimicrobial molecules in a sample of *M. scaber* collected in Benin.

Considering the activities and amounts of these two triterpenic acids in the plant, they can explain a part of the antimicrobial activity observed in the alcoholic extract against *D. congolensis*. They could also be further implicated, by their

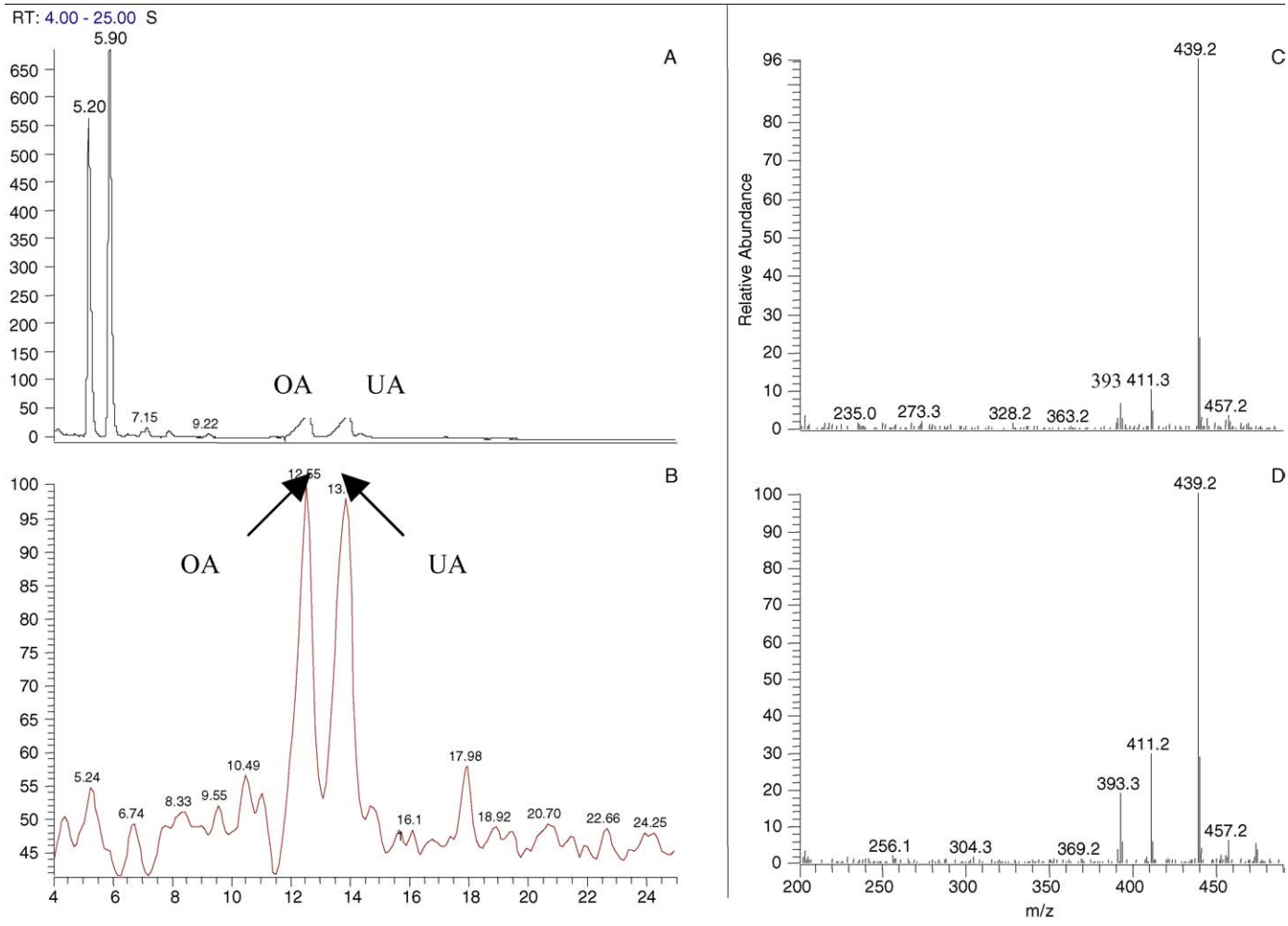

Fig. 3. LC/UV (215 nm: A) and LC/MS (TIC: B) chromatograms of standard mixture of OA and UA and full MS spectra (*m/z* 200–490) of OA (C) and UA (D) peaks.

anti-inflammatory activities, in the effects of the ointments on cattle showing dermatophilosic lesions.

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