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Comparative study of the production of coumarins and furanocoumarins in three Ruteae species

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Within specialized metabolites, coumarins and furanocoumarins represent a wide group of structurally diverse compounds and are specially produced in plants belonging to the Rutaceae family. Here we performed the furanocoumarin and coumarin-targeted chemical characterization of three Ruteae species collected from Algeria. Detection and quantification of 27 coumarins and furanocoumarins extracted from stems and leaves was carried out by UHPLC-MS. We highlighted significant chemical differences between these plants. *Ruta chalepensis* L. is the highest producer with 24.83 mmol/g dry material in stems and 15.70 mmol/g dry materials in leaves while *Haplophyllum tuberculatum (*Forsk.) is the lowest producer. We also showed a surprising chemical diversity between *R. chalepensis* L and *R. angustifolia Pers*. This chemical diversity might, therefore, be a helpful tool for phylogenetic identification of plants.

Keywords: Coumarins, Furanocoumarins, *Haplophyllum tuberculatum* (Forsk.) Juss, *Ruta angustifolia* Pers, *Ruta chalepensis* L., UHPLC-MS.

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Introduction

Rutaceae is a diverse and widely spread family of mainly tropical trees and shrubs[1]. These plants gather almost 161 genera and 1815 species distributed throughout the temperate regions of the world[2]. The most represented genera of the rutaceae family are *Citrus, Zanthoxylum*[3]*, Ruta, Ptelea, Murraya* and *Fortunella*. Both *Ruta* and *Haplophyllum* genera belong to the Ruteae tribe which gathered plants that share morphological traits such as the presence of actinomorphic, creamy-white to bright yellow flowers and specific classes of coumarins[4]. Nevertheless, *Ruta* and *Haplophyllum* show also morphological and phytochemical differences which have been described previously in literature[5,6]. In Algeria, the aerial parts of these species are often used in traditional medicine as a herbal decoction to treat various pathologies: tumours, vomiting, digestive problems and carminative, against the bites of scorpions, diabetes and infertility[7]. In other countries these plants are also used for their laxative, anti-inflammatory, anti-seizure analgesic, antipasmodic, abortifacient, antiepileptic, emmenagogue properties and for the treatment of skin diseases[8,9]. Among all the secondary metabolites produced by these plants, many reports were dedicated to coumarins and furanocoumarins[10,11]. These molecules are of greatest interest since they have a broad range of biological activities[12,13]. In plants, coumarins display important allelochemical functions and play a major role as phytoalexins in response to biotic stresses[14,15]. A recent report highlights also the involvement of coumarins in root iron homeostasis[16,17].

Many studies have focused on the beneficial effect on human health of furanocoumarins such as isoimperatorin, notopterol and bergapten[18]. These molecules possess anti-inflammatory, analgesic, anti-cancer and anti-coagulant activities[19-21]. Furanocoumarins constitute a subclass of coumarins that are characterized by the presence of an additional furan ring branched at the C6/C7 or C7/C8 position of the coumarin core molecule[22].

They have pharmacological activities as, anti-coagulant[19], anti-inflammatory[23], antioxidant[24], antimicrobial[25]. Xanthotoxin and bergapten possess photosensitising properties and are important drugs in

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the therapy of leucodermy[26]. Psoralen derivatives can inhibit DNA synthesis and may be valuable in promoting skin pigmentation and treat psoriasis[27,28]. These molecules are also involved in the Grapefruit effect by inhibiting intestinal CYP3A4 enzymes[29]. The production of these molecules is highly variable from Citrus species[30]. In this report, we compare the production of coumarins and furocoumarins in the leaves and stems of *Ruta angustifolia* Pers, *R. chalepensis* L. and *Haplophyllum tuberculatum* (Forsk.) which are three species of the Ruteae. In the present research paper, we have described the extraction, identification and quantification of 27 coumarins and furocoumarins achieved by high pressure liquid chromatography coupled with mass spectrometry (UHPLC/MS) (Fig. 1). Furanocoumarins and coumarins are secondary metabolites produced in various concentration in Rutaceae plants[10]. In this report we assessed their production in three Rutaceae collected from Algeria.

Materials and Methods

Plant materials

The aerial parts of plants were collected at flowering stage, in March 2013, from three different regions of Algeria: *R. chalepensis* L. was collected from Mostaganem (West region, 35°55′52″ North, 0°05′21″ East) at an altitude of 102 m; *R. angustifolia* Pers. From Bordj Bou Arreridj (East region, 36°4′ 0″ North, 4°46′0″ East) at an altitude of 906 m and *H. tuberculatum* from Adrar (South region, 27°52′27″ North, 0°17′37″ West) at an altitude of 257m. Plants were identified by Dr. Sekkal, from the department of Biology, University of Mostaganem (Algeria). Specimens of the 3 species (*R. angustifolia* Pers.-*1726-1, R. chalepensis* L.- 1726-2 and *H. tuberculatum* (Forsk*)* Juss-1722) were deposited in the herbarium of the Laboratory of Plant Ecology, University Ahmed Ben Bella 1 Oran (Algeria).

The sample drying was carried out in the absence of light, at room temperature. They were weighed and then grounded in liquid nitrogen using a pestle and a mortar for subsequent phytochemical investigations.

Extraction of coumarins and furanocoumarins

The extraction was performed according to the protocol described by Dugrand and collaborators with some modifications such as the volume of the methanol:water extraction solution and the composition of this solution used to resuspend the

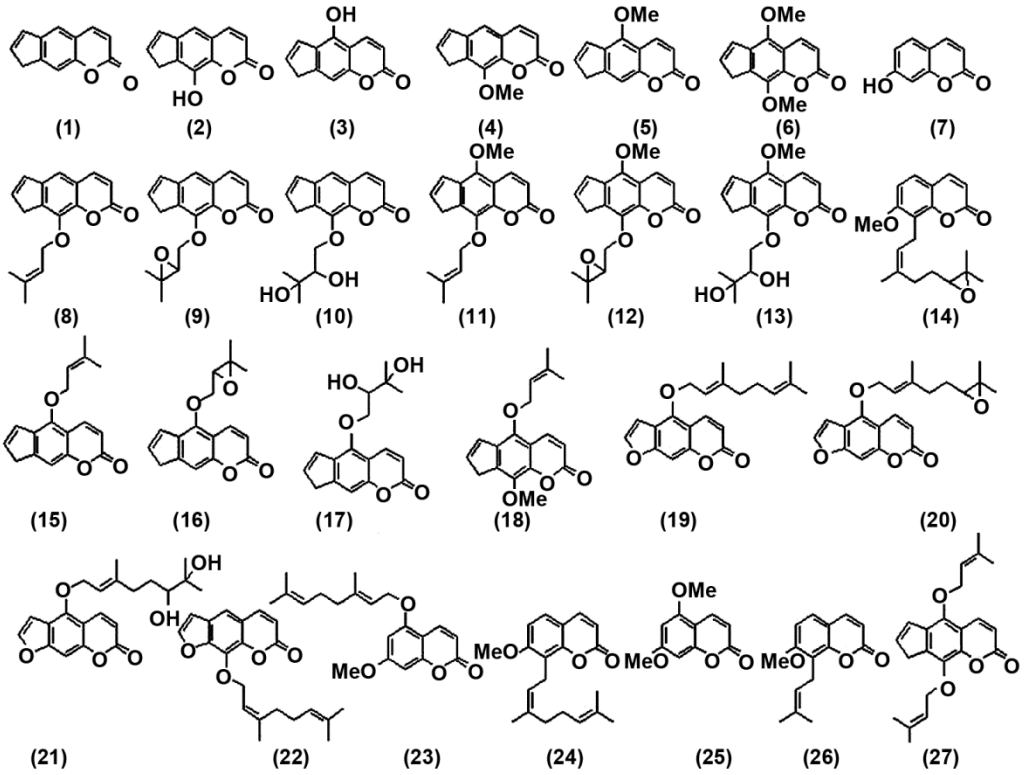

Fig. 1 — Chemical structure of the coumarins and furocoumarins analysed by UPLC/MS. The numbering corresponds to the numbering defined Table 2.

pellet after drying the methanolic extract[31]. Briefly, 20 mg of dried leaves or stems were crushed into powder in liquid nitrogen, and then 800 µL of 80:20 methanol:water solvent was added in order to solubilize the polyphenolic derivatives. Grounded material was homogenized by vortexing vigorously for 30 seconds. The samples were subsequently incubated in an ultrasonic bath (Elma, Elmasonic S70) for 15 minutes in order to increase the extraction of polyphenolics. This methanolic solution was then centrifuged at 13200 g for 30 minutes. The supernatant was recovered and a second extraction was performed on the pellet in order to complete the extraction. The final solution was dried (Concentrator Plus, Eppendorf) overnight at room temperature. The pellet was resuspended in 100 µL of 80:20 methanol:water solvant. Each extract was diluted 10 times in order to quantify molecules that were too concentrated in the stock solution[32].

Identification of furanocoumarins

The chromatographic analysis was carried out using a NEXERA HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a photodiode array (PDA), a detector (SPDM20A, Shimadzu) combined with a mass spectrometer (single quadrupole, LCMS2020, Shimadzu) according to Dugrand *et al.*[31].

Quantification of coumarins and furanocoumarins

Quantification was performed using angelicin as an analytical internal standard. This molecule is an angular furanocoumarin which is not present in the Rutaceae family. Angelicin was added at the concentration of 5 µM in the samples and in the calibration solutions. The calibration solutions contain all the molecules at the same concentrations ranging from 1 to 30 µmol/L (1, 3, 6, 12, 18, 24 and 30). The calibration curves were generated for each compound by connecting the peak intensity to the theoretical concentration of the standard in the solution. A linear regression was then performed as described elsewhere[31,32].

Statistical analysis

All experimental measurements were performed in triplicates and were expressed as mean±SD of three analyzes (means±SD).

Results

Distribution of coumarins and furocoumarins in stems and leaves

The analysis of the total amount of these molecules made evidence that *H. tuberculatum* is a poor producer of such molecules and produced them mainly in leaves (Table 1). These results are consistent with those of Diar and collaborators who reported the presence of lower amounts of psoralen in *H. tuberculatum*[33]. With 15 to 20 µmol/g dry material, *R. chalepensis* accumulates the highest concentration of these molecules whereas *R. angustifolia* has an intermediate phenotype and produces 3-4 mmol/g dry material. Interestingly, the distribution of these molecules is different between the 3 Ruteae species. For *R. chalepensis* and *R. angustifolia*, equal amounts could be detected in stems and leaves. This distribution is unequal for *H. tuberculatum*: higher in stems in leaves for *H. tuberculatum* (5.7 times more) (Table 1).

To have a better overview of the production of these molecules in plants, the authors conducted a detailed analysis of stems and leaves of 3 plants. In the light of the overall analysis done on *H. tuberculatum*, it was not surprising to highlight a very poor diversity of these molecules in the extracts. Only 5 different molecules (iepsoralen, osthol, heraclenin, aurapten and cnidicin) could be detected although at very low concentration (Table 2). This might be explained by the absence of umbelliferon which has been described to be the precursor molecule of the furanocoumarin biosynthetic pathway[34]. Consistently, umbelliferon was highlighted in *R. angustifolia* and *R. chalepensis* and both of them are producing a large diversity of downstream furanocoumarins (10 for *R. angustifolia* and 18 for *R. chalepensis*).

Phytochemical analysis

Bergaptol, xanthotoxol, byakangelicin, byakangelicol, imperatorin, phellopterin, cnidicin and 6,7-dihydroxy-bergamottin are present in *R. chalepensis* and not in *R. angustifolia*. The absence of umbelliferon in *H. tuberculatum* is however intriguing since its deriving prenylated derivatives, aurapten and osthol, could be detected in this plant. The major phototoxic

Table 1 — UHPLC/MS quantification of the 27 coumarins and furanocoumarins in the stems and leaves of the three Ruteae species.

Total coumarins and furanocoumarins (nmol/g dry matter)	*R. angustifolia*	*R. chalepensis*	*H. tuberculatum*
Stem	3685.2±1394.8	19731.7±6027.7	5.4±1.8
Leaves	3897.2±1282.4	15705.4±2666.6	30.9± 17.7

Note: The results are the means of three independent replicates and error represent standard deviation

Table 2 — Quantitative analysis of 27 coumarins/furanocoumarins by UHPLC/MS in the 3 *Ruteae*species

Cluster	Quantity coumarins and furocoumarins (nmol/g dry matter)	*R. angustifolia*		*R. chalepensis L.*		*H. tuberculatum*	
		Stem	Leaves	Stem	Leaves	Stem	Leaves
Coumarin	Umbelliferon (7)	127.4±121.9	60.2±26.6	432.7±159.2	211.2±78.5	N.D.	N.Q.
	Limettin (8)	14.5±9.4	15.6±4.6	79.0±58.8	59.3±19.1	N.D.	N.D.
	Cnidicin (19)	N.Q.	N.Q.	127.4±168.0	69.2±19.8	N.Q.	27.8±16.2
	Aurapten (22)	253.5±75.8	5.4±2.6	16.6±15.8	14.2±3.3	0.9±0.7	1.4±0.7
	Epoxyaurapten (25)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	osthol (27)	9.7±4.1	18.8±4.3	90.6±105.5	62.7±13.0	0.6±0.1	0.3±0.1
	5-Geranyloxy-7-methocycoumarin (23)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Psoralen	Psoralen (1)	445.3±196.2	112.6±36.5	4034.9±1281.9	609.8±184.7	0.2±0.1	0.7±0.3
Xanthotoxin cluster	Xanthotoxol (2)	N.D.	N.D.	44.7±40.3	37.5±8.7	N.D.	N.D.
	Xanthotoxin (4)	851.2±251.7	491.1±308.1	3050.2±842.1	1156.1±451.4	N.D.	N.D.
	Heraclenol (11)	178.0±110.6	225.3±73.9	637.5±299.9	481.1±142.1	3.0±0.7	N.D.
	Heraclenin (10)	N.Q.	N.Q.	314.9±120.1	44.8±64.2	0.7±0.2	0.7±0.4
	Phellopterin (12)	N.D.	N.D.	2291.9±530.7	N.D.	N.D.	N.D.
	Byakangelicol (13)	N.D.	N.D.	31.5±26.6	12.3±3.9	N.D.	N.D.
	Imperatorin (9)	N.D.	N.D.	382.5±66.5	91.1±53.2	N.Q.	N.Q.
	Byakangelicin (24)	N.Q.	N.D.	1046.2±215.7	801.5±175.8	N.D.	N.D.
	8-Geranyloxypsoralen (14)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Bergapten cluster	Bergaptol (3)	N.D.	N.D.	36.7±22.9	42.4±32.7	N.D.	N.D.
	Bergapten (5)	963.8±286.5	1332.7±326.7	4978.5±687.2	4262.2±55.1	N.Q.	N.Q.
	Cnidilin (15)	N.D.	N.D.	N.D.	N.D.	N.Q.	N.D.
	Bergamottin (20)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Epoxybergamottin (21)	285.6±104.9	563.3±160.1	1954.1±454.6	1976.7±300.1	N.D.	N.D.
	6,7-Dihydroxybergamottin (26)	N.Q.	N.Q.	876.1±98.9	1072.0±417.7	N.D.	N.D.
	Isoimperatorin (16)	191.6±75.6	471.2±143.5	1640.9±279.2	2208.9±246.4	N.D.	N.D.
	Oxypeucedanin (17)	253.5±75.8	452.8± 152.3	1889.4±227.5	1863.9±273.1	N.D.	N.D.
	Oxypeucedanin hydrate (18)	45.0±17.9	110.3±29.5	609.3±152.6	520.2±88.5	N.D.	N.Q.
Isopimpinellin cluster	Isopimpinellin (6)	66.1±64.4	37.9±13.7	265.2±173.7	109.2±35.3	N.D.	N.D.

Note: The numbering of the molecules corresponds to the numbering of the chemical structures. The results are the mean of three independent replicates and error represent standard deviation. N.D.= not detected, N.Q.= not quantifiable

furanocoumarins i.e. bergapten, xanthotoxin and psoralen are present in high quantities. The organ distribution of molecules is different between *R. chalepensis* and *R. angustifolia*. Regarding *R. chalepensis,* only two different groups of molecules can be observed: i) molecules which are detected nearly equal concentration in stems and leaves and ii) molecules which are present in higher amount in stems. None of them could be detected in significant higher concentration in leaves. All the molecules produced in higher concentration in stems (i.e., isopimpinellin, imperatorin, heraclenin, phellopterin, heraclenol and byakangelicol) are belonging to the xanthotoxin and the isopimpinellin clusters. Concerning *R. angustifolia,* this distribution is different. A first small group of molecules including the precursor psoralen, xanthotoxin, and aurapten are detected in higher concentration in stem. Both latest molecules also belong to the xanthotoxin cluster. The second group is composed by two coumarins (limettin and osthol) and six furanocoumarins (bergapten, isoimperatorin, oxypeucedanin, heraclenol, oxypeucedanin hydrate, epoxybergamottin). Regarding the furanocoumarins they all belong to the bergapten and the isopimpinellin cluster.

Discussion

In previous studies, classification of *Ruta* and *Haplophyllum* genera based on morphological and phytochemical traits has been contradictory. Salvo *et al.* made additional molecular analysis based on three different cDNA sequences to establish the phylogenetic relationships[4].Whereas morphological and phytochemical trees relate *Haplophyllum* and *Ruta* closely, molecular analyses confirm their distant relationship within the *Ruteae* tribe. This result is consistent with our results, since *Ruta* species are able to produce high levels of furanocoumarins while *Haplophyllum* did not. Since recent years, new precise, fast and reliable analytical methods helped

scientist to realize the chemotyping of plants and these methods became tools of choice to assess phylogenetic evolution of several plant genera. A report dedicated to the analysis of 23 eucalyptus and 64 Citrus species highlighted a distribution of total polyphenolics matching with their phylogenetic relationship[30,35]. Such a genus specific distribution of phenolics for licorice for whom the synthesis of different kind of phenolics (for example: saponins such as glycyrrhizin and glabridin; chalcones such as lichochalcone and kanzonol C; flavonoids such as liquiritigenin and kanozol D) has been reported to be species specific[36]. For Astragalus the distribution of 131 flavones, flavonols, flavanones, flavan-4-ols, isoflavones, isoflavans, petrocarpans and miscellaneous over 60 species[37] helps to identify genus with higher or lower commercial value of the roots. This report dedicated to 3 different Ruteae collected in Algeria allowed us to highlight 3 different chemotype in the same plant species. Whereas one species produces only low concentration of furanocoumarins other plants are able to synthesize significant amounts of those defense related molecules. The detailed analysis of the phytochemical profile highlighted a difference of type of molecules which are synthesized in the 3 plants. These differences might reflect 3 different branches of evolution of this genus and confirms that these molecules could be used as biochemical markers for phylogenetic studies.

Conclusion

The present study showed diversity concentrations ofcoumarins and furanocoumarins in the three Ruteae species. *R. chalepensis* L. can be considered as a good source of natural compounds. Moreover, chemotyping of furanocoumarins/coumarins could held scientists to a better understanding of plant evolution.

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