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Secondary Metabolites of *Astragalus cruciatus* Link. and Their Chemotaxonomic Significance

Wassila Benchadi[1], Hamada Haba[1*], Catherine Lavaud[2], Dominique Harakat[2] and Mohammed Benkhaled[1]

[1] *Laboratoire de Chimie et Chimie de l'Environnement (L.C.C.E), Département de Chimie, Faculté des Sciences, Université de Batna, Batna 05000, Algérie*
[2]*Laboratoire de pharmacognosie, Institut de Chimie Moléculaire de Reims, CNRS UMR 6229, BP 1039, 51097 Reims Cedex 2, France*

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Abstract: In continuation of our chemical studies on the secondary metabolites of Algerian saharan species, we report on the isolation, from the methanol extract of the whole plant *Astragalus cruciatus* Link., of seven known compounds including two saponins named azukisaponin V (**1**) and astragaloside VIII (**2**), four flavonoids called narcissin (**3**), nicotiflorin (**4**), kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**) and 5,7,2'-trihydroxyflavone (**6**) and one phytosterol glycoside, daucosterol (**7**). All the isolated compounds were characterized by using spectroscopic methods especially 1D and 2D NMR and ESI mass spectrometry and comparison with literature data. The chemotaxonomic and systematic characters of the genus *Astragalus* are summarized in this study to show its interesting chemodiversity throughout the world, as well as to establish the chemotaxonomical classification of this genus.

Keywords: Fabaceae; *Astragalus cruciatus;* Saponins; Flavonoids; Chemotaxonomic characters.

1. Introduction

Astragalus cruciatus Link. belongs to the genus *Astragalus* L. of the family Fabaceae [1]. It has four synonyms "*A. aristidis* Coss.", "*A. radiatus* Ehrenb.", "*A. trabutianus* Batt.", and *A. corrugatus* Bertol. [2,3]. The family Fabaceae, also called Leguminosae, comprises 40 tribes subdividing into approximately 750 genera and 18000 species. *Astragalus* L. is the most abundant genus of the family with an estimated 2000–3000 species and with more than 250 taxonomic sections in the world [4–6].
It belongs to the subfamily Papilionoideae or Faboideae under the subtribe Astragalinae of the tribe Galegeae [7]. The Galegeae tribe contains also three subtribes, Galiginea, Glycyrrhizinae and Coluteinae, and comprises 21 genera [4,8]. *Astragalus* plants including medicinal and poisonous species are annual or perennial herbs, small shrubs, often spiny, glabrous or hairy, hairs basifixed or bifurcate [9].

This genus is widely distributed throughout the temperate and arid regions of the world, and is principally located in Asia (1500 species), North America (500 species) and South America (150 species), and Europe (120 species), but also on mountains in Africa [10,11]. However, the center of

[*] Corresponding author: E-Mail: haba.hamada@yahoo.fr; Phone:+213-33-868980 *Fax:*+213-33-868980

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origin and biodiversity of *Astragalus* plants is Eurasia, specially the mountainous parts of South-Western and South-Central Asia [5,7,12].

Astragalus species growing in North Africa are Mediterranean or Arabian Saharan plants. They are represented by over 50 species delimited in several sections, and 15 of which are found in the Sahara of Algeria. In the flora of North Africa, 10 *Astragalus* species are endemic to Algeria, Morocco and Tunisia [1,2]. In Algeria, *A. cruciatus* Link. is locally known as «Bou akifa». This plant which is quite common in deserts and rare in Mediterranean zone belongs to the *Harpilobus* Bunge section [13]. It is an annual blue herb, 10-30 cm long, with pale yellow colored flowers, and simple hairs [1,2].

Species of *Astragalus* genus are valued in the folk medicine throughout the world and utilized as medicinal herbs against stomach ulcer, cough, chronic bronchitis, hypertension, gynaecological disorders, diabetes and venomous bites of scorpion [14]. Some plants of the same genus have been reported as having immunostimulant, cardiovascular and antiviral activities [15a,15b]. The biologically active constituents of *Astragalus* species are saponin, phenolic and polysaccharide compounds, while the toxic components consist of nitro-toxins, imidazoline alkaloids and selenium derivatives [16]. *A. cruciatus* Link., is particularly known in traditional North African medicine as a toxic species. It causes to livestock a disease called “Asaydal” [14].

The genus *Astragalus* is well known to be a rich source of bioactive secondary metabolites. Previous phytochemical investigations of various *Astragalus* species have allowed mainly the isolation and characterization of saponins [17–20] and flavonoids [21–23]. However, any constitutional or pharmacological study of *Astragalus cruciatus* Link. had never been reported.

2. Materials and Methods

2.1 General

Isolated compounds were characterized by UV (Beckman DU-600), positive and negative ESI-MS (ion trap Bruker Esquire) and extensive 1D and 2D NMR analysis (COSY, HSQC, HMBC, TOCSY, ROESY, Bruker Avance Spectrometer, ^{1}H 500 MHz, ^{13}C 125 MHz). Optical rotations were measured on a Perkin-Elmer 241 polarimeter. CC was carried out on Kieselgel 60 (320-400 mesh) and Sephadex LH-20. HPLC was performed with a Dionex apparatus equiped with an ASI-100, a P580 pump, a diode array detector UVD 170S/340S and a Chromeleon version 6.01 software, on a column interchim UP5ODB.25M, 250 × 10 mm, 5 µm. Analytical and preparative (1 mm thickness) TLCs were carried on silica gel (Kieselgel 60 F_{254}, Merck) and RP-18 (Kieselgel 60 F_{254S}) plates.

2.2 Plant material

Aerial parts and roots of *A. cruciatus* Link. were collected in May 2009 at Aïn Bennoui of Biskra vicinity of Algeria. The plant was identified by Prof. Bachir Oudjehih, Agronomic Institute of the University of Batna, where a voucher specimen, with the identification number (658/LCCE), was preserved.

2.3 Extraction and Isolation

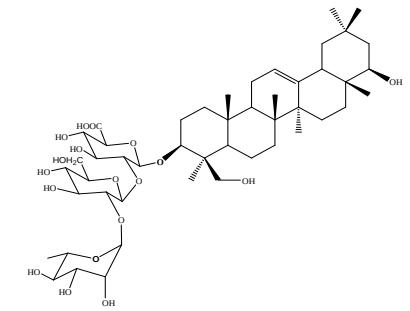
Powdered dried of whole plant (aerial parts and roots, 600 g) from *A. cruciatus* were extracted with petroleum ether (5 L × 3) during 3 days at room temperature. Filtration and evaporation to dryness gave 5 g of petroleum ether extract. The residue was extracted with ethyl acetate (5 L × 3) at room temperature during 3 days. The solvent was evaporated under reduced pressure to give 4.22 g of the EtOAc extract. The final residue was macerated in methanol (5 L × 3) under the same conditions. Filtration and evaporation to dryness yielded 43.44 g of methanol extract of which 10 g were subjected to reversed phase RP-18 vacuum liquid chromatography (VLC) using a gradient of solvents

water/methanol (80:20 to 0:100). Fractions having similar TLC profiles were pooled to afford 13 fractions (F-1 to F-13). Fraction F-8 (200 mg) containing a major component was subjected to Sephadex LH-20 CC eluting with H_2O/MeOH (20:80) to afford 6 fractions. Precipitation of the third fraction [12-30] in methanol afforded 50.7 mg of **1**. The combined fractions F-9 and F-10 (80 mg) were submitted to semi-preparative HPLC, eluting with an isocratic system (acetonitrile/water: 42:58), allowed the isolation of three pure products **2** (6.2 mg), **4** (8.3 mg) and **6** (5 mg). Fraction F-6 (235.1 mg) was applied to silica gel CC eluting with $CHCl_3$/MeOH (100:0 to 90:10) and $CHCl_3$/MeOH/H_2O (90:10:1 to 60:40:7) to obtain 8 fractions. Fractions eluted with $CHCl_3$/MeOH/H_2O (70:30:1) (19.3 mg) were purified on Sephadex LH-20 CC eluting with MeOH to provide 8 mg of **3**. Fraction F-5 (274.5 mg) was chromatographed over silica gel using a $CHCl_3$/MeOH (100:0 to 80:20) and $CHCl_3$/MeOH/H_2O (80:20:1 to 60:40:7) gradient system as eluent to yield 5 fractions. Fractions eluted with $CHCl_3$/MeOH/H_2O (70:30:3) (72.1 mg) were purified on Sephadex LH-20 CC eluting with MeOH to give 4 fractions. The second fraction [10-19] (25.2 mg) was purified by semi-preparative HPLC using a gradient of solvents acetonitrile/water: 20:80 to 45:55 to lead compound **5** (5.3 mg). Fraction F-13 was subjected to Sephadex LH-20 CC eluting with $CHCl_3$/MeOH (100:0, 99:1, 93:3, 95:5) to afford 6 fractions. Fractions (16.4 mg) eluted with $CHCl_3$/MeOH (99:1) were precipitated in methanol to obtain 5.8 mg of **7**.

3. Results and Discussion

3.1 Identification of constituents

In this study, we have isolated seven known secondary metabolites (Figure 1) including two saponins, four flavonoids and one phytosterol from the MeOH extract of *A. cruciatus*. The structures of the compounds were identified unambiguously by comparison of their ^{1}H- and ^{13}C-NMR and ESI mass spectra, and values of optical rotations with published data as azukisaponin V (**1**) and astragaloside VIII (**2**) [24], narcissin (**3**) [25], nicotiflorin (**4**) and kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**) [26], 5,7,2′-trihydroxyflavone (**6**) [27], and β-sitosterol 3-*O*-β-D-glucoside (**7**) [28].



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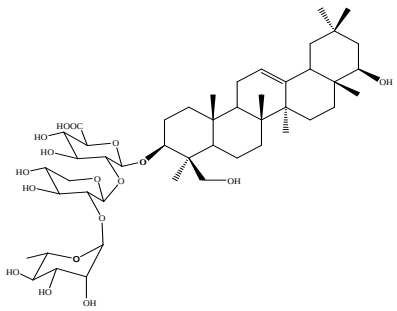

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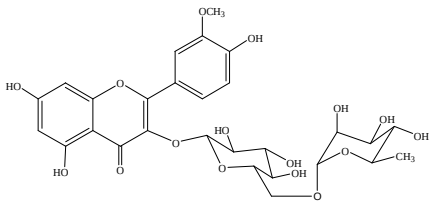

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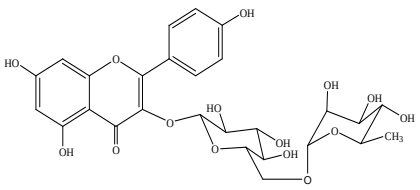
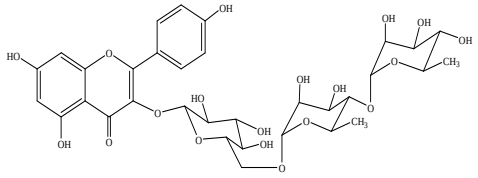


4

Secondary metabolites of *Astragalus cruciatus* link.



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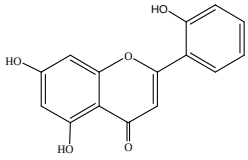

6

Figure 1. Structures of compounds **1**-**6**

3.2 Systematic characters of Astragalus genus

The genus *Astragalus* L. is well known for its worldwide taxonomic problems and is under constant revision, particularly at section level. This genus divided into two main groups Old World (Eurasia) and New World (America) is considered one of the most diverse genera of vascular plants in the world [29]. *Astragalus* L. was traditionally split into eight subgenera namely *Epiglottis*, *Trimeniaeus*, *Phaca*, *Hypoglottis*, *Calycophysa*, *Tragacantha*, *Cercidothrix* and *Calycocystis* [30]. However, taking into account the shape of leaf hairs, it has been also divided into two subgenera, simple haired subgenus of *Astragalus* (*Trimeniaeus*, *Calycophysa*, *Hypoglottis* and *phaca*) and bifurcate haired subgenus of *Cercidothrix* (*Calycocystis*, *Epiglottis* and *Cercidothrix*) [31]. In addition, the subgenera have been split into sections, 93 sections in the New World known as "Neo-Astragalus", and 152 sections in the Old World comprising 97 sections with simple hairs and 55 sections having bifurcate hairs [5,12]. The molecular phylogenetic study based on nrDNA ITS has revealed that *Astragalus* species belong to the large Astragalean clade. These species are monophyletic and form a single clade called *Astragalus s. str.* [6]. Furthermore, all annual species of *Astragalus* have been grouped in the subgenus *Trimeniaeus* and consisting of 13 sections, including sect. *Harpilobus* Bunge. This section is represented in the Mediterranean basin by many species as *A. cruciatus* Link. (syn. *A. corrugatus* Bertol.), *A. trimestris* L., *A. maroccanus* Braun-Blanq. & Maire, *A. mareoticus* Del., *A. harpilobus* Boiss. and *A. hauarensis* Boiss. [32].

*3.3 Chemotaxonomic significance of isolated saponins **1** and **2***

In this work, we have investigated the glycosidic constituents of *Astragalus cruciatus*. According to previous chemical studies, many oleanane- and cycloartane-type triterpene glycosides, flavonol glycosides and other secondary metabolites have been obtained from the Fabaceae plants [33–35]. *Astragalus* species are a source of cycloartane type saponins. These compounds are also found in Ranunculaceae, Rubiaceae, Passifloraceae, Hypoxidaceae and Combretaceae families [15a,15b]. The cycloartane glycosides isolated from *Astragalus* species were found to be diversified by their glycosylation types and linkage to the genin. This interesting chemodiversity could be used to differentiate *Astragalus* plants from each other. However, oleanane glycosides have been also isolated from *Astragalus* spp., but their occurrence is limited to skeletons common to Fabaceae family [15a]. In the present study two known triterpene saponins (azukisaponin V (**1**) and astragaloside VIII (**2**)) isolated besides four known flavonoids called narcissin (**3**), nicotiflorin (**4**), kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**) and 5,7,2'-trihydroxyflavone (**6**) and one phytosterol glycoside, daucosterol (**7**) from *Astragalus cruciatus* Link. and its chemotaxonomic relationships of other *Astragalus* species and Fabaceae family were discussed.

Literature data showed that, to date, azukisaponin V (**1**) has been isolated from different species of *Astragalus* genus (*A. flavescens* [20], *A. danicus* and *A. inopinatus* [24], *A. hamosus* [36], *A. icmadophilus* [37], *A. trigonus* [38], and *A. tribuloides* [39]) besides some other Fabaceae genus such as *Melilotus, Trifolium, Lupinus, Lathyrus, Medicago, sophora, Phaseolus* and *Vigna* [34,40–45],

while astragaloside VIII (**2**) was found in *A. complanatus* [19], *A. flavescens* [20], *A. danicus* and *A. inopinatus* [24], *A. membranaceus* var. *mongholicus* [33], *A. caprinus* [35], *A. kahiricus* [36], *A. icmadophilus* [37], *A. wiedemannianus* [46], *A. pyconocephalus* [47], and *A. angustifolius* [48] besides genus *Medicago*, *Hedysarum*, *Trifolium*, *Melilotus* and *Wisteria* [49–52]. The both compounds; azukisaponin V (**1**) and astragaloside VIII (**2**) simultaneously isolated from only four *Astragalus* species from different sections; *A. flavescens* [20] (Sect. *Eustales*), *A. danicus* (Sect. *Hypoglottis* Bunge) and *A. inopinatus* [24] (Sect. *Onobrychium*), and *A. icmadophilus* [37] (Sect. *Acanthophace*). Consequently, it is suggested that *Astragalus cruciatus* is chemotaxonomically more related to Sect. *Acanthophace*, Sect. *Eustales*, Sect. *Hypoglottis* Bunge and Sect. *Onobrychium*. These oleanane-type triterpene glycosides azukisaponin V (**1**) and astragaloside VIII (**2**) possessing a soyasapogenol B as aglycone moiety, are largely represented in higher plants of the genera of the Fabaceae family such as *Melilotus*, *Medicago*, *Trifolium* and *Lupinus*, and could be a chemotaxonomic character marker of the family including the genus *Astragalus* [40,42,49–51,53]. The presence of saponins azukisaponin V (**1**) and astragaloside VIII (**2**) makes plant material *Astragalus cruciatus* which can be used in human nutrition [50]. According to above mentioned literature data, the both saponins **1** and **2** could be a chemotaxonomic marker for *Astragalus* genus and also Fabaceae family.

3.4 Occurrence of flavonoids in Astragalus genus

Previous chemical investigations on this genus have revealed the presence of a large number of flavonol glycosides having kaempferol, rhamnetin, isorhamnetin, and quercetin skeletons [21,23,54]. The aglycones of *Astragalus* species are glycosylated by one to four sugar units which were frequently at the 3-OH position and less often at 7-OH or 4′-OH [21,55]. In this study, we have also isolated three flavonol glycosides named isorhamnetin 3-*O*-rutinoside (**3**), kaempferol 3-*O*-rutinoside (**4**) and kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**). Their structures possess isorhamnetin and kaempferol skeletons bearing bioside and trioside side chains at the C-3 position. In addition, flavonoid glycosides **3** and **4** common in *Astragalus* genus, were previously found in several *Astragalus* plants as *A. verrucosus* Moris [16], *A. dasyanthus* Pall. [56], *A. corniculatus* Bieb. [57] and *A. icmadophilus* Hand.-Mazz. [37], whereas flavonol glycoside **5** isolated from *Aceriphyllum rossii* (Oliv.) Engl. [26] and *Actinidia eriantha* Benth. [58] of the Saxifragaceae and Actinidiaceae families respectively, has been detected for the first time in the genus *Astragalus*. These results are in accordance with the chemical composition previously reported for this genus. The only flavonoid aglycone obtained herein, is 5,7,2′-trihydroxyflavone (**6**). It has been isolated from *Scutellaria* species (Asteraceae) as *S. strigillosa* Hemsl. and *S. amabilis* H. Hara [27,59]. It is very important to indicate that flavonol **5** is also found for the first time in *Astragalus* plants.

3.5 Chemotaxonomic characters of Harpilobus Bunge section

According to previous studies, taxonomy of sect. *Harpilobus* Bunge has been the subject of several revisions especially about the number of species assigned to this section [12,13]. To the best of our knowledge, the plants of this section have not been investigated chemically, except the study performed on the species *A. trimestris* L. growing in Egypt [60]. This investigation has allowed the isolation and identification of one saponin, soyasaponin I and three flavonoids called apigenin 7-*O*-β-D-glucopyranosyl-(1‴→3‴)-β-D-glucopyranoside, sorbifolin and 8-methoxyvestitol. Thus, the phytochemical constituents of both *A. cruciatus* Link. and *A. trimestris* L. (Sect. *Harpilobus* Bunge) are different in saponins and flavonoids and illustrate the chemodiversity of these species. It is noteworthy that saponin **1** azukisaponin V, the major component from this study, was previously isolated from several *Astragalus* species belonging to different sections, *A. flavescens* (Sect. *Eustales*), *A. danicus* (Sect. *Hypoglottis* Bunge), *A. inopinatus* (Sect. *Onobrychium*), *A. hamosus* (Sect. *Buceras* Bunge), *A. icmadophilus* (Sect. *Acanthophace*), *A. trigonus* (Sect. *Chronopus* Bunge), and *A. tribuloides* (Sect. *Sesamei* DC) [20,24,36-39,61].

3.6. Importance of toxic nitro compounds in Astragalus genus

The presence of toxic nitro compounds in leaflets of *A. cruciatus* Link. demonstrated previously [62] could be used to explain the toxicity of this plant to livestock [14]. These compounds which are more common among the simple haired *Astragalus* species like *A. cruciatus* Link., have been recently utilized as a tool to differentiate *Astragalus* plants to nitro-bearing and nitro-free species as well as to resolve problems of synonymy in *Astragalus* genus [63,64]. Furthermore, *A. cruciatus* Link. and *A. hamosus* L., containing similar toxic nitro compounds as miserotoxin (3-nitro-1-propyl-β-D-glycopyranoside), are taxonomically nitro-bearing species [62,63].

Although this work is a preliminary study, it seems evident that the bioactive secondary metabolites isolated from *A. cruciatus* Link. as saponins and flavonoid glycosides could be of taxonomic importance for the chemotaxonomical studies in the future, particularly in the sect. *Harpilobus* Bge., and in the Fabaceae family. This study reveals also that *A. cruciatus* Link. is chemotaxonomically more related to Sect. *Acanthophace*, Sect. *Eustales*, Sect. *Hypoglottis* Bunge and Sect. *Onobrychium*, and indicates the sectional boundary problems mostly observed in the genus *Astragalus*. The delimitation of taxa at various taxonomic ranks implies considerable taxonomic problems in the genus throughout the world. It is very important to indicate that at many places morphological characters alone are not sufficient to determine the systematic relationships among *Astragalus* species and their sections [65]. However, further phytochemical and molecular phylogenetic investigations on *Astragalus* genus are required to show particularly the relationship of *A. cruciatus* Link. with other species in the same section, and to establish clearly the chemotaxonomical classification of the genus *Astragalus*.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/RNP

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