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C-glycosylflavones in the genus *Azolla*

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ABSTRACT - Two C-glycosylflavones were isolated from the root extracts of fresh material of *Azolla filiculoides* Lam. and *A. pinnata* subsp. *africana* (Desv.) R. M. K. Saunders & K. Fowler. They were also detected in herbarium material of *A. caroliniana* Willd. and *A. nilotica* Decne. ex Mett. Their structures were assigned to homoorientin (luteolin 6-C-glucosside) and saponarin (isovitexin 7-O-glucoside). Homoorientin and saponarin have not been previously reported in the genus *Azolla.* Our results indicate that roots and fronds present a different polyphenolic composition. Previous studies and our own results point to a certain chemical homogeneity in the genus *Azolla,* with regard to polyphenols.

KEY WORDS - Azolla, Azollaceae, C-glycosylflavones, homoorientin, saponarin

Azolla Lam. is a floating freshwater pteridophyte of widespread occurrence, surviving at most latitudes. This small plant can be considered as a weed (MOORE, 1969; TEIXEIRA, 1999) but it can also have agronomic potential as a green manure ((MOORE, 1969; ASHTON & WALMSLEY, 1976; LUMPKIN & PLUCKNETT, 1980; WATANABE & VAN HOVE, 1996), because of its association with *Anabaena azollae* Stras., an N_2-fixing cyanobacterium.

These heterosporic ferns are easily influenced by environmental conditions and because of that they show a very plastic morphology. Species differentiation is mainly based on the morphology of their typical reproductive structures (PERKINS *et al.*, 1985; PEREIRA *et al.*, 1998; TEIXEIRA, 1999), which are not very common. These aspects lead to a controversial taxonomic classification. It is accepted that chemical data can help in clearing up taxonomic problems (HEYWOOD, 1973; DAHLGREN, 1980; RICHARDSON, 1989). Previous surveys in that field on different species of *Azolla* have indicated the presence of a 3-desoxyanthocyanin, luteolinidin-5-glycoside and several phenylpropanoids: caffeic and chlorogenic acids, and aesculetin (HEGNAUER, 1962; HOLST, 1977; PIETERSE *et al.*, 1977; ISHIKURA, 1982) (Table 1). In our search for polyphenols in *Azolla,* previous results pointed in the same direction (TEIXEIRA *et al.*, 1994; TEIXEIRA, 1999) (Table 1).

In the context of other studies on *Azolla* using fluorescence microscopy, we noticed some differences between fronds and roots in terms of fluorescence, which could

be related to a different chemical composition of these organs. We performed a qualitative analysis of the root extracts, from fresh and herbarium material, of *A. filiculoides* Lam. and *A. pinnata* subsp. *africana* (Desv.) R. M. K. Saunders & K. Fowler, and *A. caroliniana* Willd. and *A. nilotica* Decne. ex Mett., respectively.

According to SAUNDERS & FOWLER (1993), *A. filiculoides* and *A. caroliniana* belong to subg. *Azolla* sect. *Azolla*; *A. pinnata* to subg. *Azolla* sect. *Rhizosperma* and *A. nilotica* to subg. *Tetrasporocarpia*. By studying this material we covered four of the seven species now considered as belonging to the genus *Azolla* in order to obtain a better understanding of their polyphenolic composition.

This paper describes the techniques used and integrates the results with other phytochemical references on *Azolla*, leading to a polyphenolic profile for this genus.

MATERIAL AND METHODS

A. filiculoides was collected in Portugal, in three different places: from the basin of the Tagus river, in the centre of the country, in the basin of Guadiana river, in the south and also from the Botanical Garden of Lisbon. *A. pinnata* subsp. *africana* was collected from the Geba River, near Bafata, in the northeast of Guinea-Bissau. Voucher specimens were deposited in LISU and LISC. Herbarium material of *A. caroliniana* was obtained from LISU and *A. nilotica* from LISC.

The roots, hair-like, between 20-50 mm long, were separated from the fronds and macerated in 0.1% HCl - methanol for 24 h at room temperature. The macerate was filtered, concentrated at reduced pressure to 1/5 of its volume and refiltered. It was successively extracted with diethyl ether and EtOAc. The aqueous phase of

TABLE 1
Phytochemical references concerning polyphenols in *Azolla*. (*Product not present in *A. nilotica*).

Species	Origin	Identified compounds	References
A. imbricata *A. japonica* *A. mexicana*	—	luteolinidin-5-glycoside caffeic acid aesculetin	HEGNAUER, 1962
A. filiculoides	—	quercetin ? kampferol ?	VOIRIN, 1967
A. mexicana	Illinois, USA	luteolinidin-5-glycoside	HOLST, 1977
A. filiculoides *A. caroliniana*	Holland Holland	luteolinidin-5-glycoside apigenidin-5-glycoside	PIETERSE *et al.*, 1977
A. imbricata *A. japonica* *A. mexicana*	Japan Japan Japan	luteolinidin-5-glycoside caffeic acid chlorogenic acid p-coumaric acid quinic acid aesculetin	ISHIKURA, 1982
A. filiculoides	—	quercetin ? kampferol ?	SOEDER, 1985
A. filiculoides *A. pinnata*	Portugal Guinea-Bissau	luteolinidin-5-glycoside caffeic acid chlorogenic acid aesculetin	TEIXEIRA *et al.*, 1994
A. filiculoides *A. pinnata* *A.caroliniana* *A. nilotica*	Portugal Guinea-Bissau Portugal Moçambique	luteolinidin-5-glycoside* caffeic acid chlorogenic acid aesculetin homoorientin saponarin	TEIXEIRA, 1999

this last fraction was concentrated under vacuum, redissolved in MeOH and analysed by TLC using different solvent systems (Table 2). Spots on TLC plates were visualised under UV light after treating with NH_3 and with Neu reagent, a natural product polyethylene glycol reagent (WAGNER & BLADT, 1996). Two compounds were isolated in sufficient amount, Am1 and Am2, by preparative TLC on cellulose glass plates (Merck). Elution was carried out with HOAc-H_2O (1:9). Final purification of the product was achieved on a Sephadex LH-20 (Sigma) column by elution with MeOH.

The two compounds were identified by spectral and chromatographic comparison (TLC and HPLC) with standard products (homoorientin, Extrasynthèse 1055; saponarin, Extrasynthèse 1238), as well as by chemical evidence using acid and enzymatic hydrolysis products. UV spectra were determined on a Hitachi U-2000 spectrophotometer, using 1-cm quartz cells and MeOH as solvent (Merck).

A Perkin Elmer chromatograph was used for HPLC analysis, an LC 85B spectrophotometric detector, a LCI 100 integrator, an Lichrospher 100 RP-18 (5-mm particle size, 125x4 mm) Merck column, detection at 340 nm, and a methanol:water:acetic acid (30:68:2) mobile phase at a flow rate of 1 ml/min.

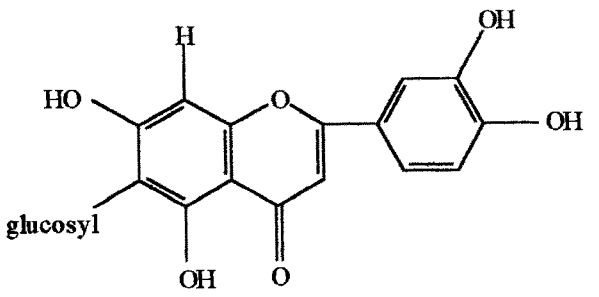

FIGURE 1 - Product Am1, homoorientin, (luteolin 6-C-glucoside).

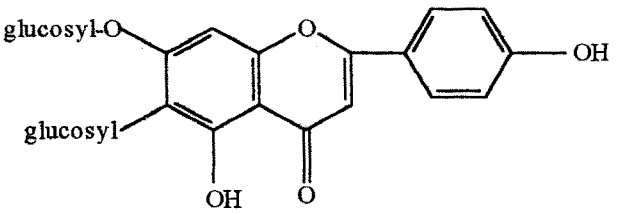

FIGURE 2 - Product Am2, saponarin, (isovitexin 7-O-glucoside).

RESULTS

Product Am1 was isolated after preparative TLC. The UV spectrum indicated a luteolin derivative: λ_{max} in MeOH (258, 270, 348 nm); NaOMe (274, 407 nm); $AlCl_3$ (276, 423 nm); $AlCl_3$-HCl (276, 383 nm); NaOAc (273.5, 376 nm); NaOAc –H_3BO_3 (262.5, 376 nm). Acid hydrolysis gave homoorientin (luteolin 6-C-glucoside) and orientin (luteolin (8-C-glucoside). The identity of Am1 as homoorientin was confirmed by co-chromatography, using TLC and HPLC, with authentic samples (Table 2 and Figure 1).

Product Am2 was isolated after preparative TLC. UV spectral data correspond to an apigenin derivative: λ_{max} in MeOH (271, 333 nm); NaOAc (272, 351, 390 nm); NaOAc-H_3BO_3 (272, 330 nm); NaOMe (269.5, 390.5 nm); $AlCl_3$ (277, 300, 350, 382 nm); $AlCl_3$-HCl (278, 300, 350, 381 nm). Partial hydrolysis by ß-glucosidase gave an unmodified product, Am2 and isovitexin (apigenin 6-C-glucoside), confirmed by co-chromatography with an authentic sample. The identity of Am2 as

TABLE 2
Chromatographic data. TLC systems: 1- cellulose plates. *n*-BuOH-HOAc-H_2O (6:1:2); 2- cellulose plates. HOAc-H_2O (1:9); Si gel plates. EtOAc-ethylmethylketone-HCOOH-H_2O (5:3:1:1)

PRODUCT	TLC SYSTEMS (R_f)			UV	NH3	NEU	HPLC R_t (min)	OBS.
	1	2	3					
Am1	0.40	0.30	0.62	violet	yellow	bright yellow	7.9	luteolin derivative
homoorientin	0.40	0.30	0.62	violet	yellow	bright yellow	7.9	reference product
Am2	0.28	0.53	0.24	dark violet	yellow	green yellow	5.9	apigenin derivative
saponarin	0.28	0.53	0.24	dark violet	yellow	green yellow	5.9	reference product

saponarin (isovitexin 7-O-glucoside) was achieved by co-chromatography, using TLC and HPLC, with authentic samples (Table 2 and Figure 2).
The presence of homoorientin and saponarin was also detected in the herbarium material of *A. caroliniana* and *A. nilotica* by TLC and HPLC.
The organic phases of the EtOAc fraction of root extracts of all plants also contained traces of the same phenylpropanoids isolated and identified in the total extracts. Anthocyanins were not found in roots.

DISCUSSION

Previous surveys on polyphenols in Azolla

Previous searches for polyphenols in the organic phases of total extracts, of both fronds and roots, of several species of *Azolla* have only indicated the presence of a 3-desoxyanthocyanin, luteolinidin-5-glycoside, and some phenylpropanoids, such as caffeic and chlorogenic acids, and aesculetin (HEGNAUER, 1962; HOLST, 1977; PIETERSE *et al.*, 1977; ISHIKURA, 1982; TEIXEIRA *et al.*, 1994), (Table 1). Later, those products were also detected following a screening of herbarium material, including *A. caroliniana* and *A. nilotica* (TEIXEIRA, 1999), (Table 1). Only *A. nilotica* does not have any anthocyanins, as previously reported (MARTIN, 1976; SAUNDERS & FOWLER, 1992).
VOIRIN (1967), working on flavonoids in the *Filicineae*, reported the probable presence of two flavonols in *A. filiculoides*, quercetin and kampferol (Table 1). SOEDER (1985), in a later study, did not mention any new compound in *Azolla* (Table 1), but only cited VOIRIN (1967). We did not find any flavonols in the studied material; instead, we identified in the root extracts two C-glycosylflavones, homoorientin and saponarin. These two compounds are reported here for the first time in the genus *Azolla*.

Biosynthetic relations

According to HARBORNE (1965; 1966), there is a biosynthetic reason for the coexistence of 3-desoxyanthocyanins with flavones and not with flavonols. A similar relation has also been observed in the *Gesneriaceae*, angiosperms possessing 3-desoxyanthocyanins but not flavonols.
Flavones might be involved in the nitrogen fixation process (PETERS *et al.*, 1986; REDMOND *et al.*, 1986; HARBORNE, 1988; HARTWIG *et al.*, 1990). In *Azolla*, nitrogen fixation occurs entirely in the upper leaf lobe cavity and there is no root nodulation. As with other polyphenols, flavones in *Azolla* might not be involved in the nitrogen fixation process, but only in the establishment of diazotrophic symbionts in the cavity (LEIZEROVICH *et al.*, 1988).
The identification of homoorientin and saponarin only in the root extracts indicates that roots and fronds in *Azolla* species present a different flavonoid composition. This might be related to the different functions performed by leaves and roots, as well as with the environmental conditions to which each organ is subjected. The floating leaves, having a prostrate habit, contain common phenylpropanoids and a variable concentration of an anthocyanin during the plant's life cycle. The submerged roots have no anthocyanins, traces of phenylpropanoids, and C-glycosylflavones.
Present results also indicate a great chemical homogeneity, with respect to polyphenols, in all the *Azolla* species studied, fresh and herbarium material and even in different populations of *A. filiculoides*. The present data complement other similar ones (HEGNAUER, 1962; HOLST, 1977; PIETERSE *et al.*, 1977; ISHIKURA, 1982; TEIXEIRA *et al.*, 1994; TEIXEIRA, 1999). The polyphenolic profile in these ferns appears to have some chemosystematic significance, as *Azolla* is the only genus in the *Azollaceae*.

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