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Full Length Research Paper

Assay of flavonoid aglycones with HPLC in four species of genus *Hypericum*

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Hypericum **plants are widely used in phytotherapy in many countries. A number of plants of this genus have exhibited various pharmacological activities attributed to their flavonoid content. The present paper describes a reverse-phase HPLC method developed for the assay of 7 flavonoid aglycones (quercetin, myricetin, isorhamnetin, rhamnetin, kaempferol, luteolin and apigenin) in polar extracts of four** ***Hypericum*** **species from UK,** ***Hypericum androsaemum*****, L.,** ***Hypericum ericoides*****, Arechav,** ***Hypericum x moserianum*** **(*Hypericum calycinum x Hypericum patulum*) Andrê and** ***Hypericum olympicum*****, L. The identity of detected flavonoids was confirmed by comparing their retention times with those of corresponding standards. The content of detected flavonoids varied from 816.69 (mg/Kg fresh wt.) in** ***Hypericum moserianum*** **to 6726.52 (mg/Kg fresh wt.) in** ***Hypericum olympicum*****. Quercetin and apigenin were present in all the species while myricetin and kaempferol were detected only in** ***H. moserianum*****.**

Key words: *Hypericum andorsaemum*, *Hypericum ericoides*, *Hypericum* x *moserianum*, *Hypericum olympicum*, flavonoids, quercetin, HPLC.

INTRODUCTION

The importance of medicinal plants to the health of individuals and communities is known since antiquity. The medicinal value of these plants lies in some chemical substances that produce a definite physiological effect on human body. The most important of these bioactive constituents of plants are alkaloids, tannins, phenolic compounds and flavonoids (Hill, 1952). Among these, flavonoids are the most ubiquitous group of plant secondary metabolites demonstrating a wide range of biochemical and pharmacological effects, including anti-inflammatory (Owoyele et al., 2008), antibacterial (Hernández et al., 2000), antifungal (Li et al., 2005), antioxidant (Bernardi et al., 2007) and anticarcinogenic (Seelinger et al., 2008).

Hypericum species are medicinal plants known as healing herbs due to their various medicinal properties for the last two hundred years (Dias et al., 1998). The whole plant extract has antidepressive effects on neurotransmitter levels in the brain (Bloomfield et al., 1996). The antidepressive (Kumar et al., 1999), anticarcinogenic (Zeng et al., 2006) and antimicrobial (Sokeman et al., 1999) activities of these plants are currently under investigation. The increasing interest in the chemistry of this genus has led to the isolation of more than 100 components with different biological activities (Quing-Li et al., 1998; Li-Hong and Keng, 2000).

Methanolic extract from the aerial parts of *Hypericum* plants typically contains hypericins, hyperforins and phenolic compounds (Barnes et al., 2001). It is especially rich in phenolics, caffeic acid, chlorogenic acid, proanthocyanidin, prenylated derivatives of phloroglucinol and flavonoids (Mojca et al., 2005). The pharmacological activity of *Hypericum* species has been linked mainly to the flavonoids and phloroglucinols (Decosterd et al., 1991; Rocha et al., 1995; Butterweck et al., 2000; Bernardi et al., 2007). Numerous flavonoids

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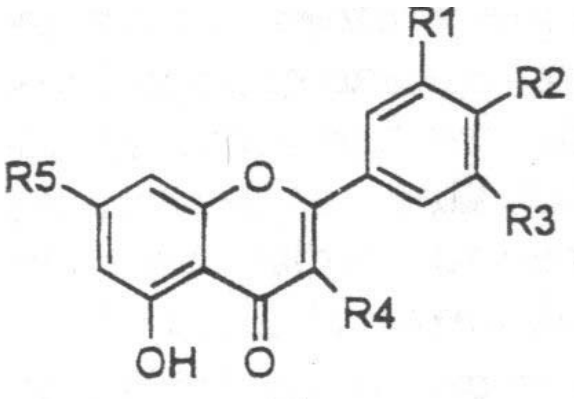
Table 1. Flavonoids isolated from genus *Hypericum*.

Flavonoid	Reference
Astraglin	Kitanov, 1988
Astilbin	Butterweck et al., 2000
3,8”-Biapigenin	Kurkin and Pradivtseva, 2007
6,8”-Diquercetin	Kurkin and Pradivtseva, 2007
Hyperoside	Kitanov, 1988; Makovetskaya, 1999
Isoquercitrin	Butterweck et al., 2000
Kaempferol	Kitanov et al., 1979; Naeem et al., 2010; Saddique et al. 2011
Miquelianin	Kitanov, 1988
Myricetin	Kitanov et al.,1979; Naeem et al., 2010; Saddique et al. 2011
Myricetin 3-glucoside	Kitanov, 1988
Myricitrin	Kitanov, 1988
Quercetin	Kitanov et al., 1979; Naeem et al., 2010; Saddique et al. 2011
Quercitrin	Kitanov, 1988; Makovetskaya, 1999
Rutin	Kurkin and Pradivtseva, 2007



Flavonoid	R_1	R_2	R_3	R_4	R_5
Quercetin	OH	H	H	OH	OH
Myricetin	OH	OH	OH	OH	OH
Isorhamnetin	OMe	OH	H	OH	OH
Rhamnetin	OH	OH	H	OH	OMe
Kaempferol	H	OH	H	OH	OH
Luteolin	H	OH	OH	H	OH
Apigenin	H	OH	H	H	H

Figure 1. Chemical structures of selected flavonoids.

have been identified in various species of genus *Hypericum* (Table 1).

Today, high performance liquid chromatography is established as the most convenient method which enables separation and identification of flavonoids using various detection systems (Janeska et al., 2007; Plazonič et al., 2009). High performance liquid chromatography methods are developed for qualitative and quantitative analyses of flavonoids in various plant materials (Bobzin et al., 2000; Dubber and Kanfer, 2004). The present paper describes a simple, precise, rapid and reproducible method to identify and quantify seven relevant flavonoid aglycones in the methanolic extracts of aerial parts of four *Hypericum* species, *Hypericum androsaemum* L., *Hypericum ericoides* Arechav, *Hypericum x moserianum* Andrê and *Hypericum olympicum* L. from UK using HPLC. The methanolic extracts were partitioned further to determine the solvent most suitable for the extraction of these flavonoid aglycones. Structures of the aglycones studied are depicted in Figure 1.

EXPERIMENTAL

Chemicals and reagents

All solvents used were purchased from Merck, Germany and were of analytical grade. Chemical standards for quercetin (98%), luteolin (95%), myricetin (95%), rhamnetin (99%), isorhamnetin (95%), kaempferol (96%) and apigenin (95%) were purchased from Sigma- Aldrich. The purity of these flavonoids was assumed as provided by suppliers and no adjustments were made in the quantitative analysis of the commercially available products. Acetonitrile, methanol and water used for HPLC analysis were HPLC grade purchased from Merck, Germany.

Plant material

The four *Hypericum* species purchased from Perryhill Nurseries were grown in the green house of University of Portsmouth, UK for one year. The aerial parts of the four species were used in the

Table 2. Percentage yield of the extracts recovered from the four *Hypericum* species.

Plant species	MeOH (%)	*n*-Hexane (%)	Chloroform (%)	EtOAc (%)	Acetone (%)	Aqueous (%)
H. androsaemum	35.50	0.96	3.41	0.84	0.83	26.10
H. ericoides	22.0	3.00	1.85	0.91	1.40	15.00
H. x *moseriarum*	20.0	1.52	0.24	0.33	0.50	18.30
H. olympicum	48.5	3.25	2.40	0.93	1.81	36.20

Table 3. Contents of flavonoids (mg/kg fresh weight) in methanolic extracts of the four *Hypericum* species.

Flavonoid	t_R (min)	*H. andorsaemum*	*H. ericoides*	*H. moserianum*	*H. olympicum*
Quercetin	2.059	405.0	855.0	9.83	48.00
Myricetin	1.787	nd	nd	540.00	nd
Isorhamnetin	2.740	10.50	13.72	nd	1038.95
Rhamnetin	3.750	383.65	4.25	nd	3622.00
Kaempferol	2.639	nd	nd	266.03	nd
Luteolin	1.966	nd	nd	0.50	4.57
Apigenin	2.487	482.0	0.67	0.33	2013.00
Sum total of flavonoids		1281.15	873.64	816.69	6726.52

t_R = retention time, nd = Not Detected.

present study. *Herbarium* specimens of all the species were lodged in the *Herbarium* of Hampshire County Council Museum Service, Winchester, Hampshire, UK (Index Herbariorum code HCMS; accession number Bi 2000 16. 370, 371, 372 and 373 for *H. androsaemum, H. ericoides, H. x moserianum* and *H. olympicum*, respectively).

Extraction procedure

For the extraction of plant material, modified method of Victória et al (2009) was used. The fresh aerial parts of the four *Hypericum* species (500 gm each) were extracted with methanol at room temperature for 24 h with occasional stirring. The methanolic extract was concentrated under reduced pressure in a rotary evaporator to give a gummy residue. The gummy residue was dissolved and suspended in water and partitioned between *n*-hexane, chloroform, ethyl acetate and acetone sequentially (three times each). These five extracts were condensed down under vacuum. The respective yields were calculated as percentage using the formula: (crude extract weight/plant material weight) x 100 and are shown in Table 2.

HPLC analysis

Preparation of standard solutions

Standard stock solutions of the reference compounds were prepared in HPLC grade methanol at a concentration of 100 µg mL^{-1} and stored in a refrigerator at -20°C until use. All standard solutions were filtered through 0.45 µm filters and diluted as necessary with methanol.

Sample preparation

For HPLC analysis a weighed amount of each extract was dissolved in HPLC grade methanol to give a concentration of 100 µg mL^{-1}. All samples were stored at 4°C and were filtered through a 0.45 µm filter before undertaking HPLC analysis.

Apparatus and Conditions

Qualitative and quantitative HPLC separation of the flavonoid aglycones in the crude extracts was performed with Waters HPLC system, equipped with a pump (1500 series), a column oven, a UV detector (2487) and a reversed-phase, pre-packed C18 column (250 x 4.6 mm, 5 µm particle size). The data was analyzed and processed using the installed Empower Software. The column was maintained at room temperature. The mobile phase was run at as a flow rate of 1.0 mL/min and consisted of acetonitrile/water 1:1 acidified with 1% acetic acid. Throughout the experiment all injection volumes were 10 µl and the compounds were detected at 254 nm. The separated flavonoid compounds were initially identified by direct comparison of their retention times with those of standards. Standard solution was then added to the sample and peaks were identified by the observed increase in their intensity. This procedure was performed separately for each standard. The flavonoid content was calculated from the peak areas of HPLC chromatograms from the 3 replicate samples.

RESULTS AND DISCUSSION

The result of qualitative and quantitative analysis of flavonoids in the four *Hyepricum* species is shown in Table 3. There are not much data on the phytochemical analysis of the four *Hypericum* species studied. This study is the first report on the flavonoid content of these species using HPLC as the chromatographic technique and shows their valuable chemical composition justifying their use in traditional medicine. The species *H. olympicum* has been shown to be richer in variety as

Table 4. Quantity of flavonoids (mg/kg fresh weight) recovered from the different extracts of the four *Hypericum* species after partitioning.

Flavonoids	*H. andorsaemum*			*H. ericoides*			*H. moserianum*			*H. olympicum*		
	EA	**AC**	**AQ**	**EA**	**AC**	**AQ**	**EA**	**AC**	**AQ**	**EA**	**AC**	***AQ***
Quercetin	nd	nd	403.00	nd	nd	852.0	nd	9.65	nd	47.42	nd	nd
Myricetin	nd	nd	nd	nd	nd	nd	nd	nd	538.01	nd	nd	nd
Isorhamnetin	10.01	nd	nd	nd	13.63	nd	nd	nd	nd	nd	3.50	1035.30
Rhamnetin	10.74	5.12	366.00	nd	4.10	nd	nd	nd	nd	nd	0.062	3620.00
Kaempferol	nd	nd	nd	nd	nd	nd	0.55	nd	264.12	nd	nd	nd
Luteolin	nd	nd	nd	nd	nd	nd	0.45	nd	nd	nd	4.45	nd
Apigenin	nd	nd	476.00	0.61	nd	nd	0.29	nd	nd	30.00	nd	1982.00

EA = ethyl acetate fraction, AC = acetone fraction, AQ = aqueous fraction.

well as in quantity of the flavonoid aglycones studied, ranging from 4.57 (luteolin) to 3622.00 (rhamnetin) (mg/Kg) of fresh plant material. Comparing the flavonoid composition of the four species reveals greater similarity among *H. androsaemum. H. ericoides* and *H. olympicum* having quercetin, isorhamnetin, rhamnetin and apigenin. Additionally, myricetin and kaempferol were found only in *H.* x *moserianum* indicating the possibility of its chemosystematic distinction.

To analyze the effect of solvent type on the extraction of flavonoids, the crude methanolic extracts of all the three species were partitioned to give non-polar (n-hexane and dichloromethane) and polar (ethyl acetate, acetone and water) fractions. The polar fractions were analyzed for their flavonoid content following the same procedure as for methanolic extract. The result of flavonoid analysis in these fractions is shown in Table 4. The result indicated that most of the flavonoids after partitioning were detected in the aqueous fraction indicating the aqueous phase to be the most effective for the isolation of flavonoid aglycones from the plant material.

Flavonoids are important secondary metabolites with widespread occurrence in plant kingdom. A number of flavonoids have shown to possess antibacterial (Harborne, 2000), anticarcinogenic (Neuhouser et al., 2004) and antiviral (Ma et al., 2001; Meragelman et al., 2001) activities.

Flavonoids occur naturally in plants as flavonoid aglycones and flavonoid glycosides.Generally, during phytochemical analysis, the extracts are hydrolysed prior to investigation for their flavonoid contents to get maximum aglycones available. However, this does not give the information regarding the aglycones naturally present in the plant material. In the present study, the plant extracts were analyzed for their flavonoid aglycones without hydrolysis to determine the aglycones naturally occurring in the four *Hypericum* species investigated. The method developed for HPLC fingerprinting provided a quick analysis of the crude extracts of the four species. The compounds were identified by comparison with the chromatogram of the seven reference standards obtained under the same conditions. The analysis time is an important factor in analytical work and the run time should be reduced to minimum in order to optimize equipment use and reduce solvent consumption. The present method gave a quick and accurate method for the separation of seven flavonoid aglycones with a run time of just 7 min.

Conclusion

The results showed that the four *Hyperiucm* species studied are a rich source of the important biologically active flavonoids. The described HPLC procedure could be useful for the qualitative and quantitative analysis of flavonoids in the genus *Hypericum*. The results might be helpful for providing chemotaxonomic relationships between species of this genus.

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