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Flavonoids and isoflavonoids from *Gynerium sagittatum*

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Abstract

Four flavonoids namely (2*R*,3*R*)-2,3-*trans*-7,4′-dimethoxydihydroflavonol, (2*R*,3*S*,4*S*)-2,3-*trans*-3,4-*cis*-7,4′-dimethoxy-3,4-flavandiol, 6-hydroxy-7,4′-dimethoxyflavone, 6,7,4′-trimethoxyflavone, along with the known isoflavonoids ferreirin, dihydrocajanin, dalbergioidin, dihydrobiochanin A and biochanin A and other 11 known compounds were isolated from the roots of *Gynerium sagittatum*. The structural characterization of these compounds was carried out via one- and two-dimensional NMR experiments in combination with ESI-MS. Finally a quantitative analysis of the isoflavones of the methanolic extract was performed by LC-ESI-MS. The high quantity of isoflavonoids found in *G. sagittatum* makes this plant a good natural source of isoflavonoids.

Keywords: *Gynerium sagittatum*; Poaceae; Flavanones; Flavandiols; Flavones; Isoflavonoids

1. Introduction

Gynerium sagittatum (Aubl.) Beauv. (Poaceae) is a tall shrub which grows in large parts of Mexico, Paraguay, Argentina and along the shores of the Peruvian Amazon (La-Torre et al., 2004) where it is known as "caña brava" and "caña isana". The infusion obtained from the roots of *G. sagittatum* has been traditionally used in the Peruvian Amazon as anti-inflammatory, analgesic and diuretic remedy whereas the infusion from the leaves is used to treat asthma and anemia (Brack Egg, 1999). Nowadays *G. sagittatum* is used as a component of an herbal medicine for the treatment of female barrenness (Li, 2006). No biological or phytochemical studies on *G. sagittatum* have been reported.

This report deals with the study of the methanol and chloroform extract of the roots which has lead to the isolation of the flavonoids (2*R*,3*R*)-2,3-*trans*-7,4′-dimethoxydihydroflavonol (**1**), (2*R*,3*S*,4*S*)-2,3-*trans*-3,4-*cis*-7,4′-dimethoxy-3,4-flavandiol (**2**), 6-hydroxy-7,4′-dimethoxyflavone (**3**) and 6,7,4′-trimethoxyflavone (**4**), along with four known isoflavanones namely ferreirin (**5**), dihydrocajanin (**6**), dalbergioidin (**7**), dihydrobiochanin A (**8**), and one known isoflavone, namely biochanin A (**9**). Additionally we isolated the known compounds, 7,4′-dimethoxyflavone (**10**), isopratol (**11**), arjunolone (**12**), tricin (**13**), tricin-5-*O*-β-D-glucopyranoside (**14**), naringenin (**15**), *Z*-cinnamic acid (**16**), *E*-cinnamic acid (**17**), 2-propenoic acid (**18**), *p*-coumaric acid (**19**) and ferulic acid (**20**). A quantitative analysis of the isoflavonoids of the methanolic extract was performed by combining high-performance liquid-chromatography diode-array detection with negative electrospray ionisation tandem mass spectrometry.

2. Results and discussion

Repeated chromatography of the chloroform and methanol extracts of the dried roots of the Peruvian plant *G. sagittatum* gave four new compounds **1–4** (Fig. 1), along with the isoflavonoids **5–9** (Fig. 2) and the known compounds **10–20**.

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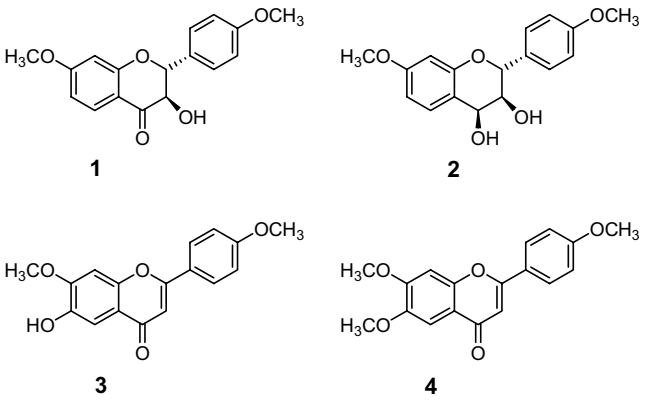

Fig. 1. Compounds **1**–**4** from *Gynerium sagittatum*.

The HR-MALDI-MS analysis of compound **1** led to the molecular peak at *m/z* 301.1006 $[M+H]^+$, which indicated the molecular formula $C_{17}H_{16}O_5$ (calcd. for $C_{17}H_{17}O_5$ 301.1076). The ESI-MS spectrum in positive ion mode showed the pseudo-molecular $[M+H]^+$ ion peak at *m/z* 301. The second order MS/MS spectrum of the parent ion at *m/z* 301 led to the significant fragment ion peaks at *m/z* 283 $[(M+H)-H_2O]^+$, *m/z* 255 $[(M+H)-H_2O-CO]^+$, *m/z* 193 $[M-C_7H_7O]^+$ (due to the loss of the B-ring) and *m/z* 151 $[(M+H)-C_9H_{10}O_2]^+$ (attributed to the retro-Diels–Alder fragmentation). This fragmentation pattern indicated the existence of a C_2–C_3 single bond and was in agreement with the established fragmentation pattern of flavanones (Chen and Montanari, 1998). The ^{1}H NMR spectrum of compound **1** showed two doublets (2H each, $J = 8.5$ Hz) of an AA′BB′ spin system at δ 7.01 and 7.51, typical of a 4′-monosubstituted B-ring of a flavonoid. Signals at δ 7.83 (1H, *d*, $J = 8.9$ Hz), 6.72 (1H, *dd*, $J = 8.9$ and 2.4 Hz) and 6.56 (1H, *d*, $J = 2.4$ Hz) have been attributed to the aromatic protons H-5, H-6 and H-8 of the A-ring, respectively. Signals of an AB system at δ 5.12 (1H, *d*, $J = 12.5$ Hz) and 4.59 (1H, *d*, $J = 12.5$ Hz) could be assigned to a *trans* diaxial H-2 and H-3 protons of the C-ring of a 3-hydroxyflavanone. The spectrum also showed the presence of two singlets corresponding to two methoxyl groups at δ 3.86 and 3.88. A ROESY experiment showed correlations between the methoxyl group at δ 3.88 and the protons at δ 7.01 (H-3′/5′), and between the methoxyl signal at δ 3.86 and the aromatic protons at δ 6.56 (H-8) and 6.72 (H-6), thus evidencing the position of the methoxyl groups at C-4′ and at C-7, respectively. The HSQC experiment provided all the connectivities between protons and their respective carbons as presented in Table 1. The HMBC experiment showed correlations between the proton signal at δ 6.56 (H-8) with two carbon resonances at δ 168.1 (C-7) and 164.6 (C-9), between the proton signal at δ 6.72 (H-6) and the carbon resonances at δ 168.1 (C-7) and 113.8 (C-10), hence confirming the position of the methoxyl group at position 7.

The absolute configuration of C-2 and C-3 carbons of compound **1** was assigned by comparison of its circular dichroism data with those of (2*R*,3*R*)- and (2*S*,3*S*)-2,3-*trans*-7,4′-dimethoxydihydroflavonols obtained by enantioselective synthesis (Van Rensburg et al., 1997).

The authors demonstrated that (2*R*,3*R*)-2,3-*trans*-7,4′-dimethoxydihydroflavonol exhibited positive and negative Cotton effects, respectively, in the 330–340 nm ($n \rightarrow \pi^*$ transition) and 285–315 nm ($\pi \rightarrow \pi^*$ transition) regions, while the (2*S*,3*S*)-2,3-*trans*-isomer showed these Cotton effects reversed in these regions. A positive $n \rightarrow \pi^*$ CE in the 330–340 nm region and a negative $\pi \rightarrow \pi^*$ CE in the 285–315 nm were observed for compound **1** and, on this basis, we ascertained its absolute configuration as 2*R*,3*R*.

Thus, compound **1** was identified as (2*R*,3*R*)-2,3-*trans*-7,4′-dimethoxydihydroflavonol, a new natural product, which might be originated from the 3,7,4′-trihydroxyflavanone (known as garbanzol), found in several plant species (Hashem, 2003; Zallocchi and Pomilio, 1994).

HR-MALDI-MS analysis of compound **2** led to the molecular peak at *m/z* 303.1218 $[M+H]^+$, which indicated the molecular formula $C_{17}H_{18}O_5$ (calcd. for $C_{17}H_{19}O_5$ 303.1232). The ^{1}H NMR spectrum of compound **2** differed from that of compound **1** only for the signals at δ 5.02 (1H, *d*, $J = 9.7$ Hz), 3.97 (1H, *dd*, $J = 9.7$ Hz and 3.6 Hz) and 4.64 (1H, *d*, $J = 3.6$ Hz) of ring C. The HSQC experiment showed key-correlations between the proton at δ 5.02 and the carbon signal at δ 77.7, between the proton at δ 3.97 and the carbon signal at 72.0, and between the proton at

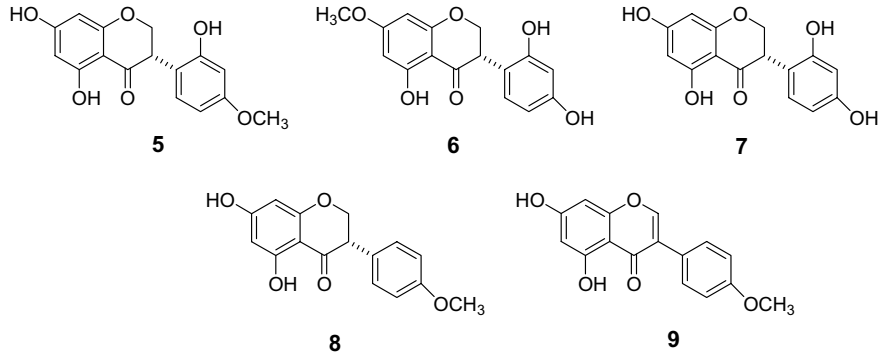

Fig. 2. Isoflavonoids from *G. sagittatum*.

Table 1
^{13}C and ^{1}H NMR data of compounds **1**–**4** in CD_3OD[a]

Position	1		2		3		4	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
2	85.5	5.12 *d* (12.5)	77.7	5.02 *d* (9.7)	165.1	–	165.5	–
3	74.7	4.59 *d* (12.5)	72.0	3.97 *dd* (9.7, 3.6)	105.0	6.77 *s*	105.5	6.82 *s*
4	194.5	–	67.4	4.64 *d* (3.6)	180.0	–	180.2	–
5	129.3	7.83 *d* (8.9)	132.5	7.26 *d* (8.0)	108.0	7.43 *s*	105.0	7.54 *s*
6	111.6	6.72 *dd* (8.9, 2.4)	108.6	6.58 *dd* (8.5, 2.4)	152.9	–	149.4	–
7	168.1	–	162.3	–	155.6	–	156.8	–
8	102.3	6.56 *d* (2.4)	101.4	6.42 *d* (2.4)	100.5	7.29 *s*	100.9	7.32 *s*
9	164.6	–	156.6	–	146.8	–	153.9	–
10	113.8	–	116.0	–	117.8	–	117.4	–
1′	129.1	–	131.0	–	124.7	–	124.7	–
2′,6′	130.3	7.51 *d* (8.5)	130.3	7.41 *d* (8.0)	128.6	8.02 *d* (8.8)	128.9	8.04 *d* (8.8)
3′,5′	114.8	7.01 *d* (8.5)	114.4	6.98 *d* (8.0)	115.1	7.13 *d* (8.8)	115.0	7.14 *d* (8.8)
4′	161.5	–	161.0	–	163.9	–	164.1	–
7-OCH_3	56.0	3.86 *s*	55.6	3.77 *s*	56.7	4.06 *s*	56.8	4.04 *s*
6-OCH_3	–	–	–	–	–	–	56.4	3.97 *s*
4′-OCH_3	56.1	3.88 *s*	55.3	3.85 *s*	55.7	3.93 *s*	55.9	3.93 *s*

[a] Assignments were confirmed by DQF-COSY, HSQC, and HMBC experiments.

δ 4.64 and the carbon signal at 67.4. The values of its C-ring three-bond proton coupling constants allowed us to propose the structure of a 2,3-*trans*-3,4-*cis*-7,4′-dimethoxy-3,4-flavandiol (**2**). The presence of a positive Cotton effect at 240 nm in the CD spectrum suggested the 2*R*,3*S*,4*S* absolute configuration for compound **2** (Ferreira et al., 2004; Slade et al., 2005). Compound **2** has been already reported only as synthetic compound (Saayman and Roux, 1965). On the other hand, 3,4,7,4′-flavantetrol (guibourtacacidin), found in *Musa sapientum* seeds (Ali and Bhutani, 1993) and *Guibourtia coleosperma* (Roux and de Bruyn, 1963), presents similar NMR data.

HR-MALDI-MS analysis of compound **3** led to the molecular peak at m/z 299.0936 $[M+H]^+$, which indicated the molecular formula $C_{17}H_{14}O_5$ (calcd. for $C_{17}H_{15}O_5$ 299.0919). The ESI-MS spectrum in positive ion mode showed the pseudo-molecular $[M+H]^+$ ion peak at m/z 299. The second order MS/MS spectrum of the parent ion at m/z 299 led to the significant fragment ion peaks at m/z 284 $[(M+H)-CH_3]^+$, m/z 256 $[(M+H)-CH_3-CO]^+$ and 597 $[2M+H]^+$. The ^{1}H NMR spectrum showed two doublets (AA′XX′ type) at δ 8.02 (2H, *d*, J= 8.8 Hz, H-2′/6′) and 7.13 (2H, *d*, $J = 8.8$ Hz, H-3′/5′) ascribable to ring B. The aromatic region also presented three singlets at δ 6.77, 7.29, and 7.43. Two singlets (3H each) were observed at δ 3.93 and 4.06, thus evidencing the presence of two methoxyl groups. The HSQC experiment correlated all protons to their respective carbons, as presented in Table 1. The HMBC spectrum exhibited correlations between the proton signal at δ 6.77 and the three quaternary carbon resonances at δ 124.7 (C-1′), 165.1 (C-2) and 180.0 (C-4), which allowed us to unequivocally assign this proton at position 3 of the flavone core. Two HMBC correlations between the singlet at δ 7.29 and the carbon resonances at δ 146.8 (C-9), and 155.6 (C-7), allowed us to assign the signal at δ 7.29 to H-8, while the proton signal at δ 7.43 showed correlations with the carbon resonances at δ 117.8 (C-10) and 152.9 (C-6) demonstrating that this proton was H-5. The ROESY spectrum showed correlations between the methoxyl group at δ 3.93 and H-3′/5′ signals at δ 7.13 allowing us to located one of the methoxyl groups at position 4′, whereas the correlation between the methoxyl signal at δ 4.06 and the aromatic proton at δ 7.29 (H-8) located the other methoxyl group at C-7. Thus, compound **3** was identified as the new compound 6-hydroxy-7,4′-dimethoxyflavone.

HR-MALDI-MS analysis of compound **4** led to the molecular peak at m/z 313.1015 $[M+H]^+$, which indicated the molecular formula $C_{18}H_{16}O_5$ (calcd. for $C_{18}H_{17}O_5$ 313.1076). The ESI-MS spectrum in positive ion mode showed the pseudo-molecular $[M+H]^+$ ion peak at m/z 313. The second order MS/MS spectrum of the parent ion at m/z 313 $[M+H]^+$ led to the significant fragment ion peaks at m/z 298 $[(M+H)-CH_3]^+$, m/z 268 $[(M+H)-CH_3]^+$ and 625 $[2M+H]^+$. A detailed analysis of the NMR data of compound **4** compared with those of compound **3** showed that the two compounds differed in the presence, in **4**, of an additional methoxyl group at δ 3.97. The presence of a ROESY correlation between the methoxyl group at δ 3.97 and the signal at δ 7.54 (H-5) evidenced that the methoxyl group was located at C-6. Thus, compound **4** was established as 6,7,4′-trimethoxyflavone. It has been reported only as synthetic compound (Bargellini and Marini-Bettolo, 1940).

The absence of the OH group in position 5 of compounds **1**–**4** makes these flavones very unusual.

We have also isolated 6,4′-dihydroxy-7-methoxyflavone (**12**), a flavone which has only been isolated once from the stem bark of *Terminalia arjuna* (Sharma et al., 1982). On the other hand, to the best of our knowledge there has been

no previous report about the occurrence of 6,7,4′-trihydroxyflavone in plants which might be an obvious precursor of these compounds.

In addition, the known isoflavonoids 5,7,2′-trihydroxy-4′-methoxyisoflavanone (ferreirin (**5**)) (Osawa et al., 1992), 5,2′,4′-trihydroxy-7-methoxyisoflavanone (dihydrocajanin (**6**)) (Osawa et al., 1992), 5,7,2′,4′-tetrahydroxyisoflavanone (dalbergioidin (**7**)) (Durango et al., 2002), 5,7-dihydroxy-4′-methoxyisoflavanone (dihydrobiochanin A (**8**)) (Heinonen et al., 2004), 5,7-dihydroxy-4′-methoxyisoflavone (biochanin A (**9**)) (de Rijke et al., 2004) have been isolated and identified by spectroscopic analysis and comparison with literature.

Furthermore 7,4′-dimethoxyflavone (**10**) (Santos et al., 1996), isopratol (**11**) (Fraishtat and Vul'fson, 1981), 6,4′-dihydroxy-7-methoxyflavone (arjunolone (**12**)) (Sharma et al., 1982), tricin (**13**) (Watanabe, 1999), tricin-5-*O*-β-D-glucopyranoside (**14**) (Adjei-Afriyie et al., 2000), naringenin (**15**), *Z*-cinnamic acid (**16**), *E*-cinnamic acid (**17**), 2-propenoic acid (**18**), *p*-coumaric acid (**19**), ferulic acid (**20**) (Agrawal, 1982) have been isolated.

Ferreirin (**5**) and dalbergioidin (**6**) were reported as phytoalexins in several plants (Ingham, 1991; Ingham and Dewick, 1984; Ingham and Tahara, 1983) while dihydrocajanin (**7**) was isolated only once from *Swartzia polyphylla* (Osawa et al., 1992).

Regarding their biological activities, compounds **5**–**8** possess antibacterial activity against cariogenic bacteria (Osawa et al., 1992), biochanin A (**9**) showed estrogenic (Pelissero et al., 1991), anti-inflammatory (Kavimani et al., 2002), anti-cancer activity in *in vitro* (Yanagihara et al., 1993) and *in vivo* assays (Lee et al., 1991). Dihydrobiochanin A was shown to be a product originated from the degradation of compound **9** by fungi of the genus

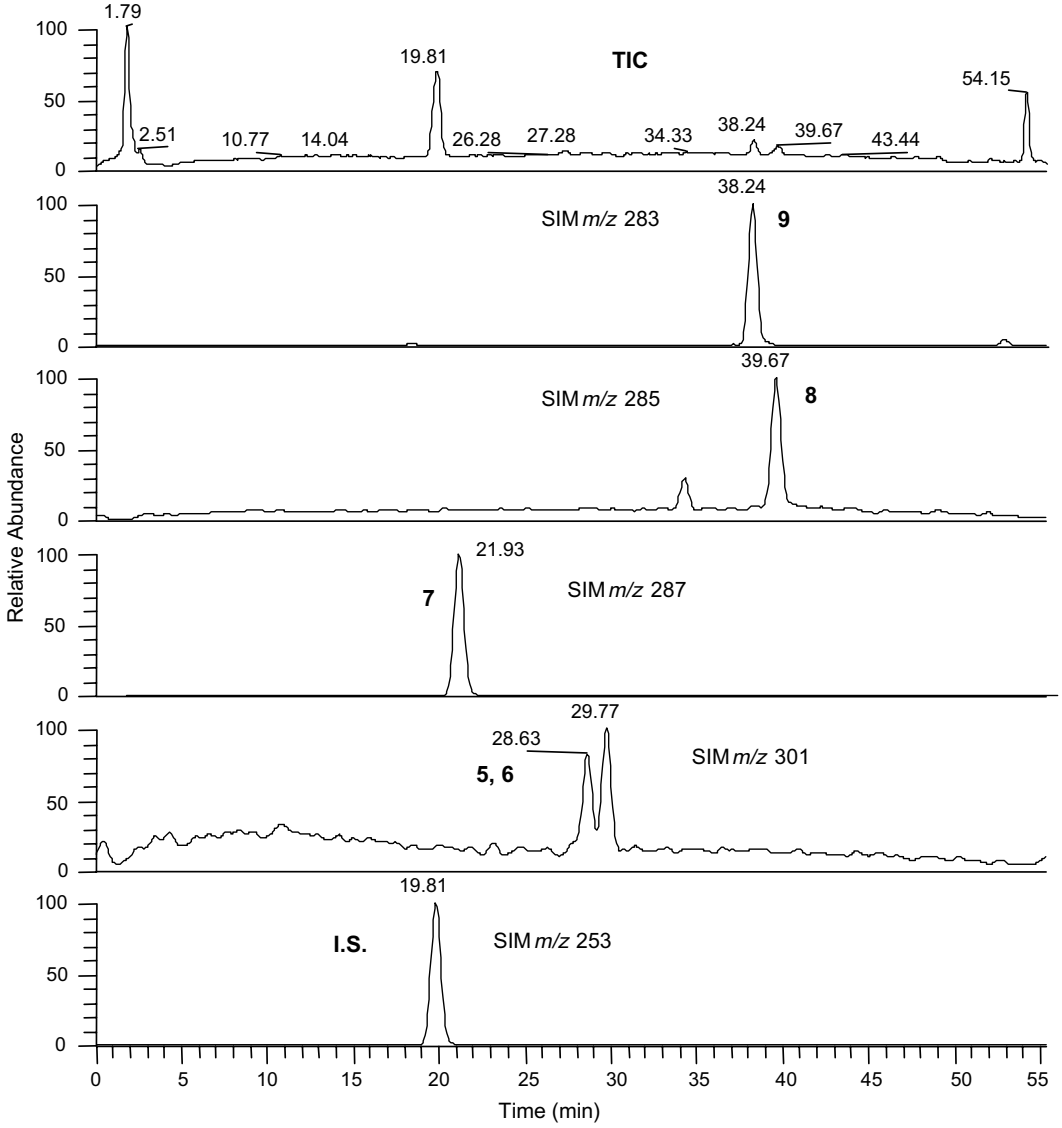

Fig. 3. TIC profile and SIM profiles of isoflavones from *G. sagittatum* methanolic extract obtained by LC-ESI-MS analysis working in negative ion mode. (**5**), Ferreirin; (**6**), dihydrocajanin; (**7**), dalbergioidin; (**8**) dihydrobiochanin A; (**9**), biochanin A, I.S., daidzein.

Fusarium (Willeke and Barz, 1982). Antifungal activity of the extract from *Swartzia polyphylla* is reported to be due to the isoflavonoids biochanin A and dihydrobiochanin A (Rojas et al., 2006).

Isoflavonoids have been shown to play a preventive role in breast, prostate and colon cancers as well as in cardiovascular diseases, osteoporosis and menopausal symptoms (Anderson et al., 1999; Tham et al., 1998; Setchell and Cassidy, 1999). Since their estrogenic-like properties have been reported in humans, their interest as phytoestrogens has grown considerably. On this basis, a quantitative analysis of these compounds in the methanolic extract of the roots of *G. sagittatum* has been performed.

The five isoflavonoids (**5**–**9**) were quantified in the methanol dried extract by LC-ESI-MS in negative ion mode. To improve the separation and identification of the single compounds, the SIM profiles of the ions at *m*/*z* 283 (**9**), 285 (**8**), 287 (**7**), 301 (**6**,**5**) were recorded. Daidzein was chosen as internal standard on the basis of its structure, its chromatographic and MS behaviour. Fig. 3 shows the TIC and the SIM profiles obtained by LC-ESI-MS of the methanol extract of *G. sagittatum*, including that of the ion at *m*/*z* 253 (daidzein).

The calibration curve obtained by plotting the area ratio between the external standards and internal standard versus the known concentration of each isoflavonoids (**5**–**9**) were linear in the range of 0.5–10 μg/ml with r^2 values of > 0.98 for all the compounds (**5**–**9**). As shown in Table 2, the major compound was biochanin A (**9**), followed by dalbergioidin (**7**), but the quantity of the other isoflavonoids (**5**, **6**, **8**) occurring in this plant was considerable too. Precision was studied by intra- and interday assays at five concentration levels for each compound. By analyzing replicate standard concentration in the same day and day-to-day, it was shown that standard deviation was not higher than 2%. Results have also shown that this analytical method was precise and reliable. Because all of the compounds are present as major constituents in *G. sagittatum*, the proposed LC-MS method may be considered as suitable for routine quantitative determination either of the plant material or its derived products, and thus may be the starting point for quality control protocols.

Isoflavones have been reported only once in the Poaceae family, specifically in *Festuca argentina*, in which the isoflavone orobol was found (Casabuono and Pomicio, 1990; Reynaud et al., 2005). Genistein, daidzein and glycitein are the most abundant isoflavone aglycones found in soy extracts, where they also occur as glycosides (da Costa et al., 2006). In red clover (*Trifolium pratense L.*) the majority of the aglycones are formononetin and biochanin A, with smaller concentrations of daidzein and genistein, the major phytoestrogens found in soy products (Wu et al., 2003). In humans, formononetin and biochanin A are metabolized to daidzein and genistein. It has been demonstrated that isoflavones from red clover significantly reduce menopausal hot flush symptoms compared with placebo (Van de Weijer and Barentsen, 2002). As a consequence of the increasing demand for new supplements, alternative isoflavonoids sources other than soy and red clover are required.

The high quantity of isoflavonoids found in *G. sagittatum* makes this plant a good natural source of these compounds and shows the potential use of this plant as an alternative to plants commonly used as nutraceutical for their high content in isoflavonoids.

Table 2
Quantitative content of isoflavonoids in dried roots from *Gynerium sagittatum*

Compound	Dried roots (mg/kg)
Biochanin A	373 ± 21
Dalbergioidin	163 ± 3
Ferreirin	41 ± 4
Dihydrocajanin	27 ± 2
Dihydrobiochanin A	15 ± 2

3. Experimental

3.1. General

NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) at 300 K dissolving all the samples in CD_3OD (Carlo Erba, 99.8%). All of the 2D NMR spectra were performed on a 512×1024 data matrix and acquired in the phase-sensitive mode with the transmitter set at the solvent resonance; time proportional phase increment (TPPI) was used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, HSQC and HMBC spectra. The NMR data were processed using UXNMR software. The ROESY spectra were acquired with $t_{mix} = 400$ ms.

ESI-MS in the negative ion mode was performed using a Finnigan LCQ Deca ion trap instrument from Thermo Finnigan (San Jose, CA) equipped with Xcalibur software. Samples were dissolved in CH_3CN:H_2O (1:1), and infused in the ESI source by using a syringe pump; the flow rate was 3 μl/min. The capillary voltage was 43 V, the spray voltage was 5 kV, and the tube lens offset was 30 V. The capillary temperature was 280 °C. Data were acquired in MS1 and MS/MS scanning mode.

LC-ESI-MS on-line analyses were performed using the same instrument described above equipped with Spectra System HPLC (Thermo Finnigan) and a DAD detector; source conditions were described above. The flow rate was 0.3 ml/min, and the postcolumn split ratio was 9:1; the capillary temperature was 280 °C.

Exact masses were measured by a Voyager DE mass spectrometer (Applied Biosystems, Foster City, CA). Samples were analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) was applied to the metallic sample plate and

dried. Mass calibration was performed with the ions from ACTH (fragment 18–39) at 2465.1989 Da and Angiotensin III at 931.5154 Da as internal standard.

HPLC separations were carried out on an Agilent 1100 series chromatograph, equipped with a G-1312 binary pump, a G-1328A rheodyne injector, a G-1322A degasser, and a G-1315A photodiode array detector using a 30 cm × 7.6 mm i.d. μ-Bondapack RP-18 column (Waters Corp., Milford, MA).

Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). All solvents for chromatographic separation were of analytical grade from Carlo Erba (Rodano, Italy).

HPLC grade methanol (MeOH), acetonitrile (CH_3CN), trifluoroacetic acid (TFA), MeOH were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ). HPLC grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA). Daidzein, used as internal standard, was purchased from Sigma Chemical Co. (St. Louis, MO).

CD data were recorded at 300 K in 1 cm rectangular pathlength cuvettes with sample volumes of 1500 μl using a JASCO J-810 spectropolarimeter. CD spectrum was collected from 350–220 nm and averaged over four accumulations using a bandwidth of 2.0 nm, sensitivity of 5 mdeg, response time of 4 s, scan speed of 50 nm/min, and step resolution of 0.1 nm. The samples were dissolved in methanol with a final concentration of 200 μM for compound **1** and 150 μM for compound **2**.

3.2. LC-ESI-MS analysis and quantification

Stock solutions of each isoflavonoid standard (1 mg/ml) were prepared by dissolving each compound in CH_3CN/H_2O (1:1). Five different solutions, containing respectively 0.5, 1.0, 5.0, 7.5 and 10 μg/ml of each compound (external standards) and 0.5 μg/ml of daidzein (used as internal standard), were prepared in CH_3CN/H_2O (1:1) and used for method development.

A solution of the dried methanolic extract was dissolved to a solution 0.1 mg/ml with CH_3CN/H_2O (1:1), added with internal standard (0.5 μg/ml) and a volume of 20 μl of the resulting solution was injected in the chromatographic system.

For quantitative purposes the extract was analyzed by LC-ESI-MS on-line using the same instruments and parameters described above. Analyses were performed on a 150 × 2.1 mm i.d. X-Terra RP-D-C_{18} column (Waters) at a flow rate of 0.2 ml/min. Gradient elution was performed with water (solvent A) and CH_3CN (solvent B). An increasing linear gradient of solvent B was used; starting at 15% B up to 100% in 60 min. Data were acquired in MS1 and SIM (single ion monitoring) scanning mode by using the mass values (pseudo-molecular ions $[M-H]^-$ of the known compounds expected to be present in the extract). In MS1 the mass range was 200–900 amu.

The quantitative determination of isoflavonoids was performed directly by LC-ESI-MS. A standard curve for each isoflavone was prepared over a concentration range of 0.5–10 μg/ml with five different concentration levels (0.5, 1.0, 5.0, 7.5 and 10 μg/ml) and triplicate injections at each level. Peak areas of the SIM spectra were used. Peak area ratios between the area of each isoflavone and that of daidzein (0.5 μg/ml) were calculated and plotted against the corresponding standard concentration using weighted linear regression to generate standard curves. Three aliquots of the extract were analyzed to quantify their isoflavonoid contents.

3.3. Plant material

G. sagittatum P. Beauv. (Poaceae) roots were collected in Iquitos, Peru, in May 2004. The plant material was identified by biologist Elsa Rengifo (Instituto de Investigaciones de la Amazonía Peruana – IIAP). A voucher specimen has been deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Universidad Nacional Mayor de San Marcos, Lima, Peru.

3.4. Extraction and isolation

Dried and powdered roots (500 g) of *G. sagittatum* were extracted for a week, three times, at room temperature using solvents of increasing polarity, namely, petroleum ether 1.5 l, chloroform 1.5 l, and methanol 1.5 l, to afford 0.63 g, 3.22 g, and 12.0 g respectively. The extractive solutions were dried in a rotavapor at 40 °C. Part of the methanol extract (3 g) was fractionated initially on a 100 × 5.0 cm Sephadex LH-20 column, using methanol as mobile phase, and 85 fractions (8 ml each) were obtained.

Fractions 39–43 and 51–56 were chromatographed by HPLC/DAD on a 300 × 10 mm i.d. μ-Bondapack RP-18 (Waters) column using H_2O/0.01%TFA as eluent A and CH_3CN/0.01% TFA as eluent B at a flow rate of 2 ml/min. For fractions 51–56 (120.0 mg) an increasing linear gradient of solvent B, starting at 30% of B up to 100% in 50 min, yielded naringenin (**15**, 2.8 mg, $t_R = 22$ min), 5,7,2′-trihydroxy-4′-methoxyisoflavanone [ferreirin (**5**), 4.5 mg, $t_R = 26.4$ min] 5,2′,4′-trihydroxy-7-methoxyisoflavanone [dihydrocajanin (**6**), 5.2 mg, $t_R = 27.7$ min], 5,7-dihydroxy-4′-methoxyisoflavone [biochanin A (**9**), 12.0 mg, $t_R = 37.1$ min] and 5,7-dihydroxy-4′-methoxyisoflavanone [dihydrobiochanin A (**8**), 5.3 mg, $t_R = 38.6$ min]. Detection was carried out at 270 nm. Fractions 39–43 (80.3 mg) was submitted to an increasing linear gradient of solvent B, starting at 15% of B up to 40% in 45 min, affording ferulic acid (**20**, 4.0 mg, $t_R = 24.3$ min), tricin-5-*O*-β-D-glucopyranoside (**14**, 7.1 mg, $t_R = 26.1$ min), arjunolone (**12**, 4.8 mg, $t_R = 29.4$ min) and *p*-coumaric acid (**19**, 8.3 mg, $t_R = 40.3$ min). Detection was carried out at 254 nm.

Fractions 62–67 and 57–61 were chromatographed by HPLC on a 300 × 7.6 mm i.d. μ-Bondapack RP-18 (Waters) column at a flow rate of 2 ml/min. Fractions

62–67 (52.0 mg) with CH_3OH/H_2O (7:3) as eluent, afforded 5,7,2′,4′-tetrahydroxyisoflavanone [dalbergioidin (**7**), 3.5 mg, $t_R = 4$ min]. Fractions 57–61 (80.0 mg) with CH_3OH/H_2O (17:8) as eluent, afforded tricin (**13**, 9.2 mg, $t_R = 8$ min).

Part of the chloroform extract (3 g) was fractionated initially on a 100×5.0 cm Silica gel H column, using hexane, ethyl acetate and chloroform as mobile phases, and 212 fractions (8 ml each) were obtained.

Fractions 73–84, 134–147, 167–172, 189–198, 199–202 were chromatographed by HPLC on a 300×7.6 mm i.d. μ-Bondapack RP-18 (Waters) column using CH_3OH/H_2O as eluent, flow rate of 2 ml/min. Fractions 73–84 (93.0 mg) with CH_3OH/H_2O (13:7), afforded a mixture of *Z*-cinnamic acid and *E*-cinnamic acid (**16** and **17**, 7.4 mg, $t_R = 12$ min), 2-propenoic acid (**18**, 5.3 mg, $t_R = 12$ min), compound **1** (3.2 mg, $t_R = 16$ min).

Fractions 134–147 (70.4 mg), purified with CH_3OH/H_2O (15:5), afforded 7,4′-dimetoxyflavone (**10**, 2.1 mg, $t_R = 16.5$ min). Fractions 167–172 (50.0 mg), purified with CH_3OH/H_2O (1:1), afforded compound **2** (2.0 mg, $t_R = 21$ min). Fraction 189–198, purified with CH_3OH/H_2O (11:9), afforded isopratol (**11**) (2.3 mg, $t_R = 29$ min) and compound **3** (3.9 mg, $t_R = 48$ min). Fraction 199–202 were separated with CH_3OH/H_2O (13:7) and afforded compound **4** (3.4 mg, $t_R = 18$ min).

3.5. (2R,3R)-2,3-trans-7,4′-Dimethoxydihydroflavonol (*1*)

Yellow powder: $C_{17}H_{16}O_5$; $[\alpha]_D^{24}$ +12.8 (MeOH; *c* 0.25); UV (MeOH) λ_{max} ($\log\varepsilon$): 273 (4.00), 315 (3.74) nm; CD (MeOH): $[\theta]_{331}$ +5285, $[\theta]_{303}$ −10762; for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; HR-MALDI-MS $[M+H]^+$ *m/z* 301.1006 (calcd. for $C_{17}H_{17}O_5$ 301.1076).

3.6. (2R,3S,4S)-2,3-trans-3,4-cis-7,4′-Dimethoxy-3,4-flavandiol (*2*)

White powder: $C_{17}H_{18}O_5$; $[\alpha]_D^{24}$ +28.1 (MeOH; *c* 0.15); CD (MeOH): $[\theta]_{232}$ +3670; for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; HR-MALDI-MS $[M+H]^+$ *m/z* 303.1218 (calcd. for $C_{17}H_{19}O_5$ 303.1232).

3.7. 6-Hydroxy-7,4′-Dimethoxyflavone (*3*)

White powder: $C_{17}H_{14}O_5$; UV (MeOH) λ_{max} ($\log\varepsilon$): 273 (3.66), 322 (3.90) nm; for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; HR-MALDI-MS $[M+H]^+$ *m/z* 299.0936 (calcd. for $C_{17}H_{15}O_5$ 299.0919).

3.8. 6,7,4′-Trimethoxyflavone (*4*)

Yellow powder: $C_{18}H_{16}O_5$; UV (MeOH) λ_{max} ($\log\varepsilon$): 273 (3.35), 324 (3.63) nm; for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1; HR-MALDI-MS $[M+H]^+$ *m/z* 313.1015 (calcd. for $C_{18}H_{17}O_5$ 313.1076).

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