Artigo

Trans-Aconitic acid, glucosylflavones and hydroxycinnamoyltartaric acids from the leaves of *Echinodorus grandiflorus* ssp. *aureus*, a Brazilian medicinal plant

*Miriam Schnitzler, Frank Petereit, Adolf Nahrstedt**

Institute of Pharmaceutical Biology and Phytochemistry, Westfaelische Wilhelms-University Muenster, Hittorfstr, 56, 48149, Muenster, Germany

RESUMO: "Ácido *trans*-aconítico, glicosilflavonas e ácido hidoxitartárico das folhas de *Echinodorus grandiflorus* ssp. *aureus, uma planta medicinal brasileira*". As folhas de *Echinodorus grandiflorus* são tradicionalmente utilizadas no Brasil devido as suas atividades diuréticas e anti-reumáticas. Com a finalidade de obter mais informações acerca de seus constituintes fenólicos, um extrato etanólico das folhas foi fracionado. Foram isolados cinco glicosilflavonas, cinco ácidos hidroxicinamoiltartáricos e o ácido *trans*-aconítico e suas estruturas foram identificadas através de dados de RMN e CLAE quiral. A determinação quantitativa dos compostos 1 - 11 através de HPLC indicou o ácido cafeoilferuloiltartárico com 0,13% como o principal representante dos ácidos hidroxicinamoiltartáricos e a swertiajaponina com 0,31% das glicosilflavonas; a quantidade de ácido *trans*-aconítico no material investigado foi de 0.98%.

Unitermos: *Echinodorus grandiflorus* ssp. *aureus*, Alismataceae, glicosilflavonas, ácido hidroxicinamoiltartárico, ácido *trans*-aconítico.

ABSTRACT: The leaves of *Echinodorus grandiflorus* are used traditionally in Brazil for their diuretic and antirheumatic activities. In order to obtain more information about its phenolic constituents, an ethanol leaf extract was fractionated. Five glucosylflavones, five hydroxycinnamoyltartaric acids and *trans*-aconitic acid were isolated and their structures identified by NMR data and chiral HPLC. Quantitative determination of compounds 1 - 11 by HPLC indicated caffeoylferuloyltartaric acid with 0.13% as the main representative of the hydroxycinnamoyltartaric acids and swertiajaponin with 0.31% of the glucosylflavones; the content of *trans*-aconitic acid in the investigated material was 0.98%.

Keywords: *Echinodorus grandiflorus* ssp. *aureus*, Alismataceae, glucosylflavones, hydroxycinnamoyltartaric acids, *trans*-aconitic acid.

INTRODUCTION

Echinodorus grandiflorus ssp. *aureus* (Fassett) R. R. Haynes & Holm. Niels. (Alismataceae), is a perennial plant, generally found in canals, marshes and at lake shores in the southeast of Brazil. The leaves are used in traditional Brazilian medicine for their diuretic and antirheumatic properties (Tanaka et al., 1997; Agra et al., 2007). Recently, a methanol extract of *E. grandiflorus* was found to have antimicrobial, analgesic and anti-inflammatory activity (De Souza et al., 2004; Dutra et al., 2006). Unpublished data by Cechinel Filho (Univali, Itajai-SC, Brazil) identified caffeic acid, ß-sitosterol, and the flavones swertisin and swertiajaponin as constituents. The presence of the cembrane diterpenes echinodol (Manns; Hartmann, 1993) and echinoic acid (Tanaka et al., 1997) has also been discussed. The present study on further constituents was initiated because phytochemical knowledge about medicinally used plant material should be as broad as possible for a rational basis of its therapeutic usage.

MATERIAL AND METHODS

Plant material

Leaves of the plant were collected in September (spring time), 2001 in Ilhota, Santa Catarina State, Brazil and air-dried. A voucher specimen has been deposited at the herbarium of the Institute of Pharmaceutical Biology and Phytochemistry, Westfaelische Wilhelms-University Muenster, under the number PBMS 199.

Extraction and fractionation

The pulverized, air-dried leaves (1 kg) were submitted to an ultra-turrax extraction with CH_2Cl_2 to remove lipids. The residue (956 g) was exhaustively

extracted with EtOH 70% (v/v) to yield 202 g after lyophilization (total extract). The dry EtOH extract was dissolved in water and partitioned with EtOAc (A) and a second time after acidifying with HCl to pH of 2.0 (B). The EtOAc-fractions were evaporated (residue of A: 11.5 g; of B: 13.3 g) and used for further purification.

Chromatographic systems

System 1: MPLC on MCI®-gel CHP-20P (2.5 x 50 cm), BESTA E100 pump, linear MeOH/water gradients; detection by TLC.

System 2: MLCCC (Ito Multilayer Coil Separator-Extractor, P.C., Inc. Potomac, MD/USA); column: 325 mL, I.D. 1.6 mm; Knauer HPLC Pump 64, rotation: 800 rpm, FWD; flow 1 mL/min, 3 mL/tube; detection by TLC.

System 3: Preparative HPLC (Eurospher 100, RP-18, 7 mm, 20 x 250 mm; Waters multisolvent delivery system 600; Waters 490 Multiwavelength Detector; l = 280 nm; mobile phase: A = Acetonitrile, B = 0,1% TFA in Aqua Millipore®.

System 4: MPLC column (RP-18, 18-32 mm, 100 Å, 36 x 500 mm); Besta Technik; BESTA E100 pump; linear MeOH/water gradients; detection by TLC.

TLC: Aluminum sheets with 0.2 mm SiO_2 (Merck, Darmstadt) developed with EtOAc/MeOH/H_2O/HCOOH 100:10:10:1; detection at 254 and 360 nm and by Naturstoff reagent (diphenylboryloxyethylamine 1% in MeOH).

Fractionation of the neutral EtOAc phase (A)

The EtOAc phase A (11.5 g) was fractionated by MPLC on a polyamide column (25 x 500 mm, packed with 75 g polyamide SC 6; Knauer HPLC Pump 64; Gradient: Aqua dem. 1.0 L, MeOH 25% 0.5 L, MeOH 50% 1.0 L, MeOH 75% 0.5L, MeOH 100% 1.0 L; flow 7 mL/min, 28 mL/tube) resulting in three fractions. Phenolic constituents were detected by TLC in fractions two and three.

Fraction two (tubes 57-106) was further fractionated by MPLC on system 1 with MeOH 10% → 80%, 2.0 L each; flow 8 mL/min; 24 mL/tube) to give five subfractions. Subfraction 3 (tubes 107-121) was further fractionated by system 2 with *n*-heptane/EtOAc/MeOH/H_2O (1:19:1:19), mobile phase was the upper phase. Pure isovitexin (**11**, 15 mg) was obtained from the eluate of 570 to 990 mL.

Fraction three (tubes 107-136) was further fractionated using the system 1 with the same solvent as above with 7 mL/min and 21 mL/tube) to give eight fractions. Fraction 7 (tubes 133-135) yielded pure swertisine (**7**, 58 mg); fraction 8 (136-148) was submitted to system 3 with a linear gradient of 20 % to 23 % A in B within 5 min followed by isocratic flow; 8 mL/

min). The peak at 24 min yielded pure isoorientin-7,3´-dimethylether (**8**, 5 mg).

Fractionation of the acidic EtOAc phase (B)

Phase B (13.3 g) was fractionated by system 2 with CHCl$_3$/MeOH/H_2O/*n*-PrOH (10:10:6:1, mobile phase was the upper phase) resulting in 9 fractions. Phenolic constituents were detected in fractions 1-5 and 9.

Fraction one of MLCCC (tubes 36-50) was submitted to system 4 with a linear gradient of MeOH 20% (0.8 L) → 80% (1.0 L); flow 8.4 mL/min; 12.6 mL/tube) and gave three subfractions. Subfraction 1 (tubes 40-58) was submitted to system 1 with a linear gradient of MeOH 10% → 80%, 1.25 L each; flow 6 mL/min; 24 mL/tube) yielding pure 2-*O*-caffeoyltartaric acid (**2**, caftaric acid, 152 mg; 204-282 mL). Subfraction 3 of MPLC (tubes 88-115) was submitted to system 1 with the same eluent system as for subfraction 1 yielding pure isoorientin (**9**, 81 mg; 522-552 mL) and swertiajaponin (**10**, 30 mg; 576-588 mL).

Fraction two of MLCCC (tubes 51-60) was fractionated by system 4 with a linear gradient of MeOH 10% → 80%, 1.5 L each; flow 7.2 mL/min; 22 mL/tube) and gave eight subfractions. Subfraction 1 (tubes 20-33) was subfractioned by system 1 using a linear gradient of MeOH 10% → 80%, 1.0 L each; flow 7 mL/min; 14 mL/tube) yielding pure *trans*-aconitic acid (**1**, 795 mg; 532 – 620 mL). Subfraction 6 (tubes 73-93) was further fractionated by system 1 with a linear gradient of MeOH 10% → 80%, 1.5 L each; flow 7 mL/min; 28 mL/tube) yielding pure dicaffeoyltartaric acid (**4**, 370 mg, chichoric acid; 1944 - 2968 mL). Subfractions 3 and 4 (tubes 61-80) separated by system 1 with a linear gradient MeOH 10% → 80%, 1.5 L each; flow 6 mL/min; 18 mL/tube) and yielded pure 2-*O*-feruloyltartaric acid (**3**, 75 mg; 1124 - 1540 mL).

Fraction five of MLCCC (tubes 81-110) was submitted to a Sephadex® LH-20 column (2.3 x 75 cm), mobile phase EtOH 96%, Flow 1.0 mL/min, 10 mL/tube; detection by TLC. The combined fraction in tubes 49-65 was lyophilized and further fractionated by system 3 isocratically with 25 % A in B at a flow of 4 mL/min. The peak eluting at 42 min was further purified by system 3 isocratically with 22% A in B. The peak at 60 min yielded pure caffeoyl-feruloyltartaric acid (**5**, 50 mg).

Fraction nine of MLCCC (tubes 161-198) was fractionated by system 1 with a linear gradient of MeOH 10% → 80%, 1.5 L each; flow 6 mL/min; 18 mL/tube). The fraction eluting at 2142 – 2468 mL was lyophilized and was further purified by system 3 with a linear gradient of 20 % A to 45 % A in B within 20 min, flow 10 mL/min.). The peak at 22 min. yielded pure diferuloyltartaric acid (**6**, 13 mg).

Because the NMR data of compounds **5** and **6**

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are not fully reported in the literature, they are presented here as a complete set:

Caffeoyl-feruloyl-(2R,3R)-(+)-tartaric acid (**5**): $[\alpha]^{20}_D$ -214 (*c* 0.055; MeOH); ^{1}H-NMR (400 MHz, CD$_3$OD, δ_H 3.35 ppm): δ 5.79 (2H, *s*, H-2 and H-3), 7.70/7.22 (2H, *d*, *J* 1.9/1.9 Hz, H-2′ and H-2′′), 6.77/6.81 (2H, *d*, *J* 8.2/8.2, H-5′ and H-5′′), 6.97/7.10 (2H, *dd*, *J* 1.8/8.2; 1.8/8.2, H-6′ and H-6′′), 7.64/7.70 (2H, *d*, *J* 15.9/15.9, H-7′ and H-7′′), 6.35/6.47 (2H, *d*, *J* 15.9/15.9, H-8′ and H-8′′), 3.88 (OCH$_3$); ^{13}C-NMR (100 MHz, CD$_3$OD, δ_C 49.3 ppm): δ 169.73 (C-1 and C-4), 72.78 (C-2 and C-3), 127.60/127.60 (C-1′ and C-1′′), 115.27/111.80 (C-2′ and C-2′′), 146.89/149.43 (C-3′ and C-3′′), 150.00/150.96 (C-4′ and C-4′′), 116.62/116.58 (C-5′and C-5′′), 123.49/124.62 (C-6′′ and C-6′′), 148.53/148.42 (C-7′ and C-7′′), 113.76/114.17 (C-8′ and C-8′′),167.67/167.67 (C-9′ and C-9′′), 56.69 (OCH$_3$); ESI-MS *m/z* 487.1 [M-H]⁻, 975.0 [2M-H]⁻.

Di-feruloyl-(2R,3R)-(+)-tartaric acid (**6**): $[\alpha]^{20}_D$ -189 (*c* 0.013; MeOH); ^{1}H-NMR (400 MHz, CD$_3$OD, δ_H 3.35 ppm): δ 5.85 (2H, *s*, H-2 and H-3), 7.27 (2H, *d*, *J* 1.9 Hz, H-2′ and H-2′′), 6.85 (2H, *d*, *J* 8.2, H-5′ and H-5′′), 7.15 (2H, *dd*, *J* 1.9/8.2, H-6′ and H-6′′), 7.75 (2H, *d*, *J* 16.0, H-7′ and H-7′′), 6.51 (2H, *d*, *J* 16.0, H-8′ and H-8′′), 3.93 (OCH$_3$); ^{13}C-NMR (100 MHz, CD$_3$OD, δ_C 49.3 ppm): δ 169.73 (C-1 and C-4), 72.78 (C-2 and C-3), 127.62 (C-1′ and C-1′′), 114.19 (C-2′ and C-2′′), 148.41 (C-3′ and C-3′′), 150.99 (C-4′ and C-4′′), 116.59 (C-5′ and C-5′′), 124.65 (C-6′′ and C-6′′), 149.46 (C-7′ and C-7′′), 111.80 (C-8′ and C-8′′), 167.65 (C-9′ and C-9′′), 56.71 (OCH$_3$); ESI-MS *m/z* 501.0 [M-H]⁻; 1002.9 [2M-H]⁻.

Chiral HPLC of tartaric acid

To determine their absolute configuration, the tartaric acid derivatives were submitted to alkaline hydrolysis as described by Becker and Hsieh (1985) followed by chiral HPLC on a Chirex (D)-penicillamine column (4.6 x 50 mm, Phenomenex, Aschaffenburg, Germany) of thus obtained tartaric acid: isocratic system with 85% of a solution of 1.0 mM copper (II) acetate and 0.05 M ammonium acetate in water, pH 4.5, and 15% isopropanol at 60 ºC. Detection was at 280 nm. T$_R$ of (2*S*,3*S*)-D-(-)-tartaric acid was 1.3 min. and of (2*R*,3*R*)-L-(+)-tartaric acid 8.7 min.

Quantitative HPLC

Preparation of the extract: 2 g of the pulverized drug material was extracted with 30 mL CH$_2$Cl$_2$ under ice cooling using an Ultra-Turrax (IKA, Staufen, Germany) to remove lipids (e.g. chlorophylls). The residue was extracted three times with EtOH 70% under the same conditions. The collected EtOH phases were concentrated by rotation and finally freeze dried; the drug:extract

residue was 4:1. For quantitative HPLC, 20.0 mg of the extract were dissolved by sonication in 10.0 ml MeOH/ Aqua Millipore (1:1) and filtered through a membrane filter. For quantification three extracts were prepared.

Calibration: The glucosylflavones were determined as isoorientin at 270 nm and the hydroxycinnamoyltartaric acids as caffeoyltartaric acid at 290 nm using methylcinnamate as an internal standard. Isolated isoorientin (r^2 = 0.9995) and caffeoyltartaric acid (r^2 = 0.9984)) were used for calibration. Factor for correction determined by HPLC at the wavelength chosen was 0.296 for the glucosylflavones, 0.265 for the hydroxycinnamoyltartaric acids with one chromophor (**2**, **3**) and 1.297 for those with two chromophores (**4** - **6**). *Trans*-aconitic acid was determined at 220 nm using an external calibration curve (r^2 = 0.9989). For intraday and interday precision three extracts were prepared at day 1 and at the following two days and quantified; the overall relative standard deviation was ± 3.65%.

HPLC System: Waters Multisolvent Delivery System 600 with Waters 515 HPLC pump; Waters 990 Photodiode Array Detector; detection at 290, 280, 270 and 220 nm. Column: Luna C-18, 5μm, 150 x 4 mm (Phenomenex, Aschaffenburg, Germany); mobile phase A = AcCN, B = 0.1% TFA in Aqua Millipore®: 0-20 min 1% A : 99% B; 20-45 min 20 : 80; 45-50 min 50 : 50; 50-55 min 100 : 0; 55-68 min 1 : 999; flow 0.6 mL/min. Assignment of peaks was done by their online UV spectra and after spiking them with the isolates **1** - **11** (Fig. 2).

RESULTS AND DISCUSSION

Fractionation of a defatted ethanol extract from the air-dried leaves was primarily conducted to obtain more information about the phenolic constituents of *E. grandiflorus* ssp. *aureus*. We isolated 2-*O*-caffeoyltartaric acid (**2**), 2-*O*-feruloyltartaric acid (**3**), cichoric acid (**4**), caffeoyl-feruloyl-tartaric acid (**5**), di-feruloyl-tartaric acid (**6**), swertisin (**7**), isoorientin-7,3′-dimethylether (**8**), isoorientin (**9**), swertiajaponin (**10**) and isovitexin (**11**) and the non-penolic *trans*-aconitic acid (**1**) (see Fig. 1). An analytical HPLC of a crude 70% ethanol extract is presented in Fig. 2; it shows the good separation of all major constituents **1** - **11**. All except compounds **5** and **7** (Cechinel Filho, pers. comm.) were isolated for the first time from this plant material.

Structures were elucidated by spectroscopic methods (^{1}H and ^{13}C NMR data, 1D- and 2D NMR experiments, ESI-MS and optical rotation) on comparison with data in the literature for flavone-*C*-glycosides (Davoust et al., 1980; Kato; Morita, 1990), tartaric acid derivatives (Singleton et al., 1978; Cheminat et al., 1988; Mulinacci et al., 2001), and *trans*-aconitic acid (Cai et al., 2001). The absolute configuration of the tartaric acid was determined by chiral HPLC to be the L-(+)- enantiomer (2*R*,3*R*-enantiomer).

Quantitative determination (Table 1) of the

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flavone-C-glycosides resulted in a total of 0.6% with swertiajaponin (0.31%; **10**) as the main compound. The hydroxycinnamoyltartaric acid esters sum up to 0.9% with cichoric acid (dicaffeoyltartaric acid, 0.42%; **4**) as the main representative; in any case, (*2R,3R*)-tartaric acid is realized without any isomerization product. The highest yield with almost 1% was for *trans*-aconitic acid (**1**). However, it should be noticed that the quantitative data were obtained from one sample only.

Interestingly, only flavone 6-C-glycosides have been detected in the leaves of *E. grandiflorus* ssp. *aureus*; there was no indication of the accumulation of flavonols, O-glycosides or 8-C-glycosides. C-glycosilated flavones seem to be widely distributed in the Alismataceae family; they do not only occur in a close relative, *E. muriaticus*, but also in related genera such as *Alisma, Baldellia, Damasonium* and *Sagittaria* (Hegnauer, 1986). With

respect to their tartaric acid esters, the leaves of *E. grandiflorus* ssp. *aureus* qualitatively and quantitatively resemble the herb of *Echinacea purpurea* (Soicke et al., 1988; Bauer et al., 1988).

trans-Aconitic acid occurs occasionally in higher plants although data on its distribution are rare. Nierhaus and Kinzel (1971) investigated 31 species of 27 families of higher plants for *trans*-aconitic acid as its TMS derivative by gas chromatography. Only species of the Ranunculaceae and Gramineae showed *trans*-aconitic acid as "main organic acid" or "in higher concentration" (absolute data are not given); all others showed "traces" only. The content of *trans*-aconitic acid in *E. grandiflorus* ssp. *aureus* with almost 1% is in a mean range of higher plants; thus the title plant does not belong to the accumulators of *trans*-aconitic acid which can reach up to 11% of dry weight as was shown for *Asarum europaeum*
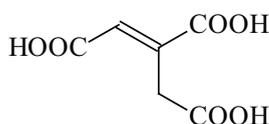
(**1**) *trans*-aconitic acid
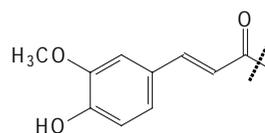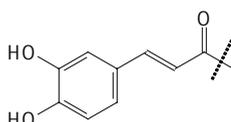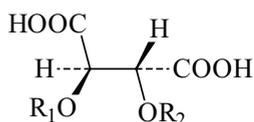
R₁=R₂=H: (2*R*, 3*R*)-(+)-tartaric acid

caffeoyl

feruloyl

		R₁	**R₂**
(**2**)	2-*O*-caffeoyl-(2*R*,3*R*)-(+)-tartaric acid	caffeoyl	H
(**3**)	2-*O*-feruloyl-(2*R*,3*R*)-(+)-tartaric acid	feruloyl	H
(**4**)	dicaffeoyl-(2*R*,3*R*)-(+)-tartaric acid	caffeoyl	caffeoyl
(**5**)	caffeoyl-feruloyl-(2*R*,3*R*)-(+)-tartaric acid	caffeoyl	feruloyl
(**6**)	diferuloyl-(2*R*,3*R*)-(+)-tartaric acid	feruloyl	feruloyl

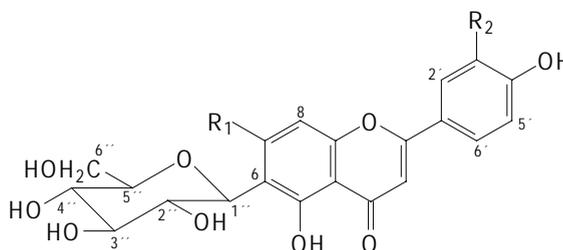|		**R₁**	**R₂**
(**7**)	swertisin	OMe	H
(**8**)	isoorientin-7,3'-dimethylether	OMe	OMe
(**9**)	isoorientin	OH	OH
(**10**)	swertiajaponin	OMe	OH
(**11**)	isovitexin	OH	H

Figure 1. Compounds isolated from the leaves of *E. grandiflorus* ssp. *aureus*.
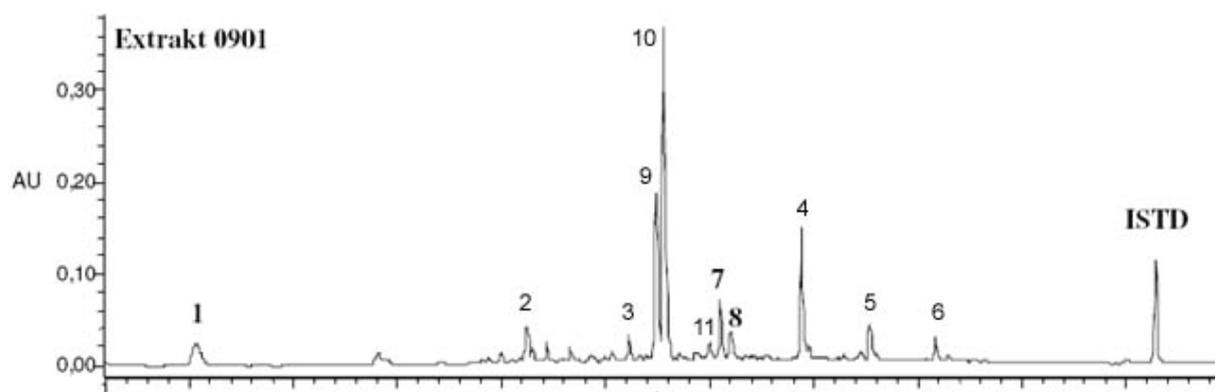
Figure 2. HPLC of the 70% ethanol extract for quantification from the dried leaves of *E. grandiflorus* ssp. *aureus*; detection shown here: 270 nm; chromatographic conditions see *2.4*. Numbering of peaks as in Figure 1; ISTD = Internal standard (methylcinnamate).

Table 1. Contents of compounds **1 - 11** in % of the crude dried leaves of *E. grandiflorus* ssp. *aureus* harvested in Sept. 2001. **1** was determined using an external standard curve; **2 - 6** were determined as caffeoyltartaric acid with a correction factor used for the disubstituted hydroxycinnamoyltartaric acids; **7 - 11** were determined as isoorientin. Mean values and their deviation are from three independently processed samples.

trans-Aconitic acid	**(1)**	0.977 ± 0.028	
2-*O*-Caffeoyltartaric acid	**(2)**	0.133 ± 0.009	
2-*O*-Feruloyltartaric acid	**(3)**	0.112 ± 0.004	
Dicaffeoyltartaric acid	**(4)**	0.423 ± 0.043	
Caffeoylferuloyltartaric acid	**(5)**	0.130 ± 0.011	
Diferuloyltartaric acid	**(6)**	0.105 ± 0.007	Sum 0.90
Swertisin	**(7)**	0.066 ± 0.009	
Isoorientin-7,3'-dimethylether	**(8)**	0.033 ± 0.003	
Isoorientin	**(9)**	0.154 ± 0.030	
Swertiajaponin	**(10)**	0.308 ± 0.052	
Isovitexin	**(11)**	0.033 ± 0.005	Sum 0.59

(Krogh, 1971). Bohman et al. (1983) found *trans*-aconitic acid from cereal pastures with up to 5% responsible for tetany in cattle; the toxic product is not compound **1** itself but tricarballylic acid that is produced by reduction of **1** by the microflora in the rumen (Schwartz et al., 1988). Grunes (1967) argued that *trans*-aconitic acid higher than 1% already may cause tetany in the animals; thus the leaves of *E. grandiflorus* ssp. *aureus* may be toxic for ruminants. Compound **1** also shows allelopathic (Voll et al., 2005) and antifeeding activity (Nagata; Hayakawat, 1998); thus the amount of almost 1% *trans*-aconitic acid in *E. grandiflorus* ssp. *aureus* may serve for its chemical defense. The Material Safety Data Sheet (https://fscimage. fishersci.com/msds/73684.htm) informs that pure *trans*-aconitic acid in man causes eye, skin and gastrointestinal irritation; it also states that no toxicological information was found; the leaves of *E. grandiflorus* ssp. *aureus* can therefore be regarded as non-toxic for man. On the other hand, *cis*-aconitic acid, which is a metabolic product of the citric acid cycle, inhibits carcinogenesis induced by 3,4-benzopyrene in experimental animals (Kallistratos; Kallistratos, 1976), but *trans*-aconitic acid has not been investigated.

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