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Artigo

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

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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

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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 subfractionated 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**

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_3OD, 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_3OD, 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_3OD, 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_3OD, 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_2Cl_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.

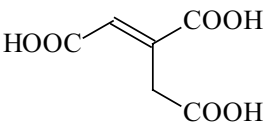
Structures were elucidated by spectroscopic methods (^{1}H and ^{13}C NMR data, 1D- and 2D NMR experiments, ESI-MS and optical rotation) on comparison with data in the literature for flavone-*C*-glycosides (Davoust et al., 1980; Kato; Morita, 1990), tartaric acid derivatives (Singleton et al., 1978; 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

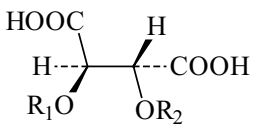
flavone-C-glycosides resulted in a total of 0.6% with swertiajaponin (0.31%; **10**) as the main compound. The hydroxycinnamoyltartaric acid esters sum up to 0.9% with cichoric acid (dicaffeoyltartaric acid, 0.42%; **4**) as the main representative; in any case, (*2R,3R*)-tartaric acid is realized without 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).

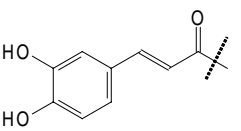
trans-Aconitic acid occurs occasionally in higher plants although data on its distribution are rare. Nierhaus and Kinzel (1971) investigated 31 species of 27 families of higher plants for *trans*-aconitic acid as its TMS derivative by gas chromatography. Only species of the Ranunculaceae and Gramineae 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*



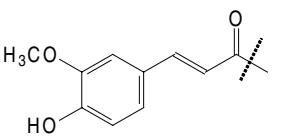
(**1**) *trans*-aconitic acid



R_1=R_2=H: (2*R*, 3*R*)-(+)-tartaric acid



caffeoyl



feruloyl

		R_1	R_2
(**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

		R_1	R_2
(**7**)	swertisin	OMe	H
(**8**)	isoorientin-7,3'-dimethylether	OMe	OMe
(**9**)	isoorientin	OH	OH
(**10**)	swertiajaponin	OMe	OH
(**11**)	isovitexin	OH	H

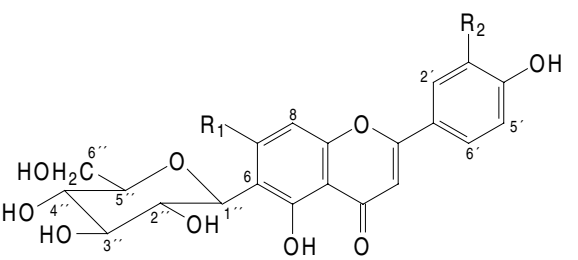


Figure 1. Compounds isolated from the leaves of *E. grandiflorus* ssp. *aureus.*

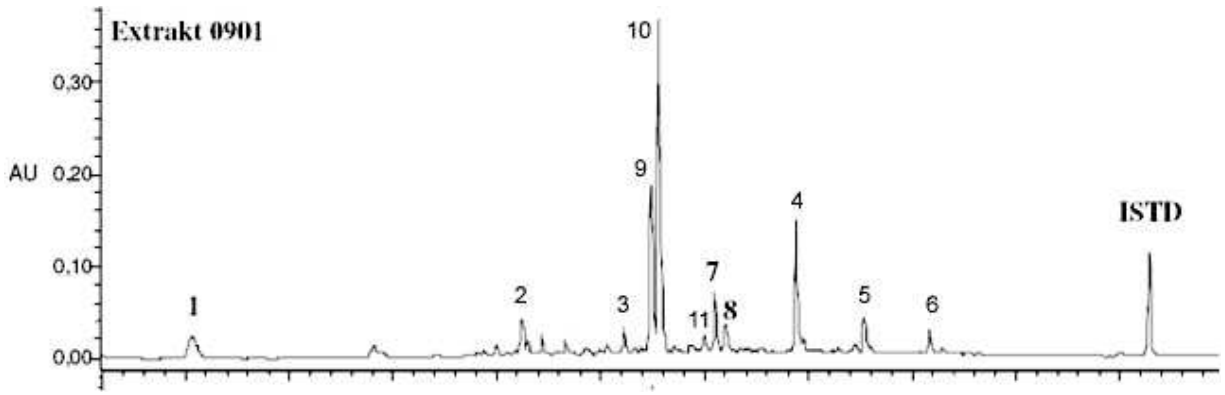


Figure 2. HPLC of the 70% ethanol extract for quantification from the dried leaves of *E. grandiflorus* ssp. *aureus*; detection shown here: 270 nm; chromatographic conditions see *2.4*. Numbering of peaks as in Figure 1; ISTD = Internal standard (methylcinnamate).

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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Toxicological evaluation by in vitro and in vivo assays of an aqueous extract prepared from *Echinodorus macrophyllus* leaves

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Abstract

Toxicity of an aqueous extract prepared from *Echinodorus macrophyllus* dried leaves, a plant used in folk medicine to treat inflammation and kidney malfunctions, was estimated by different bioassays. Mutagenicity of the aqueous extract was evaluated in the *Salmonella*/microsome assay (TA97a, TA98, TA100 and TA102 strains), with or without metabolic activation. No mutagenic activity (lyophilized extract tested up to 50 mg/plate) could be detected to any of the tester strain. Furthermore, no cytotoxic effect has been observed when a crude extract of *E. macrophyllus* (up to 7.5 mg/ml) was tested on the exponential growth of hepatoma and normal kidney epithelial cells in culture. Toxicity of *E. macrophyllus* was also evaluated in male Swiss mice after 6 weeks of continuous ingestion of the aqueous extract in drinking water. Average daily ingested doses were 3, 23 and 297 mg/kg for a lyophilized extract, and 2200 mg/kg for a crude extract, with dose two being equivalent to the daily dose recommended to humans. At the end of the treatment, all animals revealed a deficit in final body weight ranging from 5 to 47%. Biochemical

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BPB, bromophenol blue; DMEM, Dulbecco's Minimal Essential Medium; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; NaOH, sodium hydroxide; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; Tris, Tris(hydroxymethyl)aminomethane.

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analysis of the plasma revealed some minor alterations indicating subclinical hepatic toxicity. Genotoxic effect on liver, kidney and blood cells has been also evaluated by the comet assay, being negative to liver and blood cells. However, DNA analyses of the kidney cells detected some genotoxic activity for the highest dose tested of *E. macrophyllus* extract, either lyophilized or crude. On the other hand, exposure dose of 23 mg/kg, equivalent to the daily dose recommended to humans, did not revealed any genotoxic effect and hence this herb seems to be safe to human organism.

Keywords: *Echinodorus macrophyllus*; Aqueous extract; Genotoxicity; Comet assay; Mutagenicity

1. Introduction

The use of plants for healing purposes is getting increasingly popular as they are believed as being beneficial and free of side effects. However, most of the information available to the consumer about several medicinal herbs does not have any scientific data support. Medicinal herbs have their use as medicament based simply on a traditional folk use that has been perpetuated along several generations. One example of a very poorly studied medicinal plant is *Echinodorus macrophyllus*, an aquatic plant from the Alismataceae family, indigenous to the American continent. Although more than 40 species have been catalogued to the genus *Echinodorus*, only two are used in folk medicine, *E. macrophyllus* and *Echinodorus grandiflorus*, both known in Brazil as 'chapéu de couro'. Both species have been used as diuretic, anti-arthritic, anti-nephritic, anti-lithiasis, and anti-rheumatic medicinal agent. Partial phytochemical identification has been described only for *E. grandiflorus* (Manns and Hartmann, 1993; Tanaka et al., 1997; Costa et al., 1999). To date, no studies have been performed about the toxicological potential of both medicinal *Echinodorus* species to humans. Therefore, preliminary toxicological investigation of *E. macrophyllus* has been performed in male Swiss mice with special attention to a genotoxic effect on liver, kidney and blood cells. In vitro bioassays were also performed to determine its cytotoxic and mutagenic potentials.

2. Material and methods

2.1. Chemicals

Chemicals used in the mutagenicity assay were of the highest purity (Sigma (St Louis, MO) or Merck (Brazil)), including 4-nitroquinoline 1-oxide, 2-aminofluorene, 2-aminoanthracene, benzo[a]pyrene, sodium azide, dantron, hydrogen peroxide (Perhydrol 30%), and DMSO (dimethylsulfoxide). Dulbecco's Minimal Essential Medium (DMEM), penicillin, streptomycin, trypsin, Triton X-100, and bromophenol blue were purchased from Sigma (St Louis, MO). Fetal bovine serum (FBS) was from Cultilab (Campinas, Brazil). Ethidium bromide was supplied by Serva (Heidelberg). Ca^{2+} and Mg^{2+} free Hanks balanced salt solution, EDTA (ethylenediaminetetraacetic acid), and agarose were from GibcoBRL (Gaithersburg, MD). All other reagents were of analytical grade purchased from Merck (Brazil).

2.2. Plant material

Dried leaves of *E. macrophyllus* were kindly provided by a local pharmaceutical industry (Laboratório Simões, Rio de Janeiro, Brazil). Plant material was originally collected in the vicinity of Carangola, Minas Gerais, Brazil, and a voucher sample of the same (No. 84807) was deposited at the herbarium Bradeanum (Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil).

2.3. Herb extracts preparation for the bioassays

Aqueous crude extract of *E. macrophyllus* dried leaves was prepared as follows: for each gram of leaves, 10 ml (for in vitro assays) or 100 ml (for animal assays) of boiling water were added, left cooling at room temperature and filtered. For lyophilization, 20 ml of hot extracting water were used for each gram of herb. The yield of lyophilized residue corresponded to 13.5% (13.5 g of residue for each 100 g of original dry leaves), being stored at −20°C, until further use.

2.4. Bacterial strains and mutagenicity assay

The *Salmonella typhimurium* strains TA97a, TA98, TA100, and TA102 were used in this study. The experimental protocol adopted has been introduced by Maron and Ames (1983), with minor modifications (Gomes et al., 1995). Briefly, 100 μl aliquots of lyophilized *E. macrophyllus* aqueous extract in deionized water, at concentrations ranging from 1 to 500 mg/ml, were mixed to 100 μl of each bacteria strain (10^9 cells/ml) and 500 μl of 0.1 M sodium phosphate buffer, pH 7.4. After 30 min of incubation at 30°C, with shaking, 2 ml of top agar (46°C) were added and the mixture poured onto minimal glucose agar plates. After incubation for 72 h, at 37°C, the number of revertant *his*$^+$ bacteria colonies was scored. In experiments with metabolic activation, 500 μl of S9 mixture (lyophilized rat liver S9 fraction from Moltox™ (Molecular Toxicology, Annapolis, USA)) were added to the mixture of bacteria and *E. macrophyllus* extract and processed as described above. Suitable positive controls were included in each assay (Table 1). Mutagens were dissolved either in DMSO or deionized water. Assays were performed in triplicate and data shown corresponds to the mean of two independent determinations. Standard deviations of the mean did not exceed 15% among each group of triplicate plates.

Table 1
Mutagenicity of the lyophilized aqueous extract of *E. macrophyllus* dried leaves in *S. typhimurium* TA97a, TA98, TA100, and TA102, without (−S9) and with (+S9) metabolic activation

Dose (μg/plate)	Revertants per plate[a]							
	TA97a		TA98		TA100		TA102	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
50 000	61 ± 4	–	44 ± 2	–	241 ± 5	–	341 ± 98	–
40 000	194 ± 79	–	40 ± 2	–	185 ± 15	–	261 ± 42	–
30 000	290 ± 53	–	39 ± 14	–	204 ± 41	–	219 ± 31	–
20 000	177 ± 15	–	41 ± 4	–	100 ± 8	–	194 ± 16	–
10 000	77 ± 40	–	39 ± 4	–	212 ± 36	–	230 ± 43	–
5000	161 ± 22	171 ± 16	38 ± 6	58 ± 11	186 ± 9	151 ± 10	259 ± 32	213 ± 62
4000	–	204 ± 26	–	61 ± 13	–	168 ± 4	–	213 ± 65
3000	–	210 ± 35	–	58 ± 10	–	176 ± 14	–	290 ± 63
2000	–	196 ± 13	–	66 ± 4	–	140 ± 22	214 ± 30	254 ± 52
1000	112 ± 8	205 ± 40	41 ± 5	61 ± 5	199 ± 6	115 ± 17	256 ± 35	289 ± 51
500	–	183 ± 13	–	66 ± 12	–	144 ± 10	276 ± 17	241 ± 23
100	–	178 ± 16	–	54 ± 15	–	141 ± 21	260 ± 22	280 ± 44
0	125 ± 27	201 ± 11	44 ± 6	69 ± 2	190 ± 16	154 ± 6	247 ± 50	302 ± 49
Positive controls[b]	695 ± 177	728 ± 78	562 ± 43	179 ± 35	875 ± 40	410 ± 4	515 ± 25	846 ± 73

[a] Mean ± standard deviation.
[b] Positive controls: TA97a, 4-nitroquinoline 1-oxide (1μg; −S9) and 2-aminofluorene (5μg; +S9); TA98, 4-nitroquinoline 1-oxide (2μg; −S9) and 2-aminoanthracene (2,0 μg; +S9); TA100, sodium azide (1μg; −S9) and benzo[a]pyrene (2μg; +S9); TA102, hydrogen peroxide (200μg; −S9) and dantron (30μg; +S9).

2.5. Mammalian cell culture and cytotoxic assay

Rat hepatoma cells (HTC cell line, BCRJ No CRO22), and human embryo kidney epithelial cells (HEK cell line, BCRJ No CR017), were purchased from Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). Cells were routinely grown in DMEM supplemented with 10% FBS and antibiotics, and maintained in 10% CO_2, at 37°C, using standard cell culture techniques. For cytotoxicity assay, cells were plated onto 35 mm diameter culture dishes (Nunc) in DMEM/10% FBS and 24 h later, growing medium was renewed and crude aqueous *E. macrophyllus* extract added to different concentrations. Adherent cells were collected daily by in situ fixation with 5% TCA and their number determined by protein staining with bromophenol blue (BPB). For this, fixed cells were stained for 30 min with 1% BPB in 1% acetic acid, rinsed three times with water and stain extracted for 15 min with 10 mM unbuffered Tris base. Absorbance of extracted BPB was determined at 570 nm (Microplate reader, BioRad).

2.6. Animals treatment

Male adult Swiss mice (25–35 g), were used in this investigation. Groups of five to seven animals were fed ad libitum with a commercial rodent diet (Nuvilab Ltd, Curitiba, Brazil) and free access to drinking water containing *E. macrophyllus* aqueous extract. Three different doses of the lyophilized extract (group 1, 1.25 mg%; group 2, 12.5 mg%, and group 3, 125 mg%) and one dose of the crude extract (group 4, 1000 mg%) were tested. Animals receiving water were used as normal control. Solutions of drinking water containing the herb were always fresh prepared and changed every day. The volume of consumed water was daily recorded to calculate the average amount of *E. macrophyllus* extract ingested per animal per day. After 6 weeks of treatment, animals were anaesthetized lightly with anaesthetic ether and killed by cardiac puncture. Animals were not fasted before blood collection. Plasma was collected immediately by centrifuging heparinized blood and kept at -20°C for further biochemical determinations.

2.7. Biochemical determinations

Plasma samples were analyzed for determination of total protein, albumin, globulins, creatinine, and urea nitrogen (Mega bioanalyzer, Merck), and for enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALA), and alkaline phosphatase (ALP) (Cobas Integra bioanalyzer, Roche), using appropriate kits supplied by the manufacturers.

2.8. Tissue cellular dissociation

Liver and kidney cells were dissociated by mincing a small piece of the organs into very fine fragments, in ice cold dissociation solution (Ca^{2+} and Mg^{2+} free Hanks balanced salt solution supplemented with 20 mM EDTA) and the suspension left to settle for a couple of minutes to allow sedimentation of large debris.

2.9. Single cell gel electrophoresis assay (SCGE) (comet assay)

The alkaline version of the comet assay detects single and double strand breaks in DNA (Fairbairn et al., 1995). To detect these lesions, 10 μl of liver or kidney cell suspension or 10 μl of heparinized whole blood were mixed with 120 μl of 0.5% low-melting-temperature agarose in PBS and added to microscope slides pre-coated with 1.5% normal-melting-temperature agarose in PBS. Slides were covered with a microscope coverslip and refrigerated for 5 min to gel, followed by immersion in ice-cold alkaline lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% DMSO, 1% Triton X-100, final pH >10.0) for at least 1 h. Slides were then incubated for 20 min in ice cold electrophoresis solution (0.2 M NaOH, 1 mM EDTA), followed by electrophoresis at 25 V/300 mA, for 25 min. After electrophoresis, slides were rinsed with water, allowed to dry at 37°C and stained with 20 μg/ml ethidium bromide. DNA of individual cells was viewed using an epifluorescence microscope (Olympus), with 516–560 nm emission from a 50-W mercury light source, and quantitated as described below.

2.10. Quantitation of DNA lesions

Quantitation of DNA breakage was achieved by visual scoring of 50 randomly selected cells per slide, classifying them into five categories representing each different degrees of DNA damage, ranging from no comet (type 0, undamaged cells) to maximum length comet (type 4, maximally damaged cells). Comets of type 1 are representative of cells with a minimal detectable frequency of DNA lesions while comets of type 2 and 3 are representative of cells with moderate low to moderate high frequency of DNA lesions, respectively. Cellular comets were classified by investigators blinded to the experimental conditions of the treated animals from which the tissue samples were obtained.

2.11. Statistics

The results from in vivo experiments were statistically evaluated by Student's *t*-test.

3. Results and discussion

3.1. Mutagenicity evaluation of E. macrophyllus in S. typhimurium

Since no documented results are available about the toxicological potential of *E. macrophyllus*, studies have been initiated by us to elucidate this question. Toxicological studies were concentrated on an aqueous extract prepared from *E. macrophyllus* leaves since this is the most popular preparation form of this herb. Toxicity of the herb was estimated by in vivo and in vitro bioassays. Mutagenic potential of *E. macrophyllus* was investigated in the *S. typhimurium* microsomal activation assay. Evaluations were performed with the lyophilized extract, on different *S. typhimurium* strains (TA97a, TA98, TA100 and TA102), in the presence and absence of S9 mixture. As shown in Table 1, no mutagenic activity could be detected in either condition, with concentrations tested up to 50 mg of lyophilized extract per plate. Similar results were obtained when a second bacterial mutation assay was used, the SOS-chromotest (Quillardet et al., 1982), as described elsewhere (Valsa et al., 1990; Asad et al., 2000) (data not shown). Absence of mutagenic activity had been already observed by Rivera et al. (1994), when they screened several Brazilian medicinal plants, including *E. macrophyllus*, for the presence of potential genotoxic activity.

3.2. In vitro evaluation of the cytotoxic potential of E. macrophyllus

Studies were performed in rat hepatoma cells (HTC cell line), as liver cells are pivotal in general metabolism control and in human embryo epithelial kidney cells (HEK cell line), as kidney cells are potentially targeted by substances present in *E. macrophyllus* leaves. Exponential growth of both cell lines was followed for 5–6 days in the absence and presence of *E. macrophyllus* crude aqueous extract and the resulted growth kinetics are shown in Fig. 1 (A and B). A differentiated growth behavior has been observed for each cell type when challenged with doses up to 7.5 mg/ml of the extract. On hepatoma cells, an increasing dose related growth inhibition has been observed without any parallel signs of cell death (Fig. 1A) Conversely, when epithelial kidney cells were grown in the presence of *E. macrophyllus* extract, a clear dual growth effect could be detected (Fig. 1B). Doses up to 1 mg/ml of the extract were growth stimulatory, changing afterwards to an increasing dominant inhibitory growth effect. Concomitant DNA synthesis analysis performed in the first 72 h of the growth stimulation of HEK cells revealed that *E. macrophyllus* did not affect the incorporation of thymidine into DNA (data not shown). As the cytotoxic assay used in this study is based on protein mass staining, it might be that the stimulatory effect observed on HEK cells was the consequence of an increase in cell size without an increase in cell number, a biologic event known as hypertrophy. Since kidneys are commonly associated with compensatory hypertrophy (Preisig, 1999), it might be that *E. macrophyllus* aqueous extract does contain substances that somehow could modulate renal hypertrophy, a conclusion also justified by the specificity of the stimulatory effect, since it has

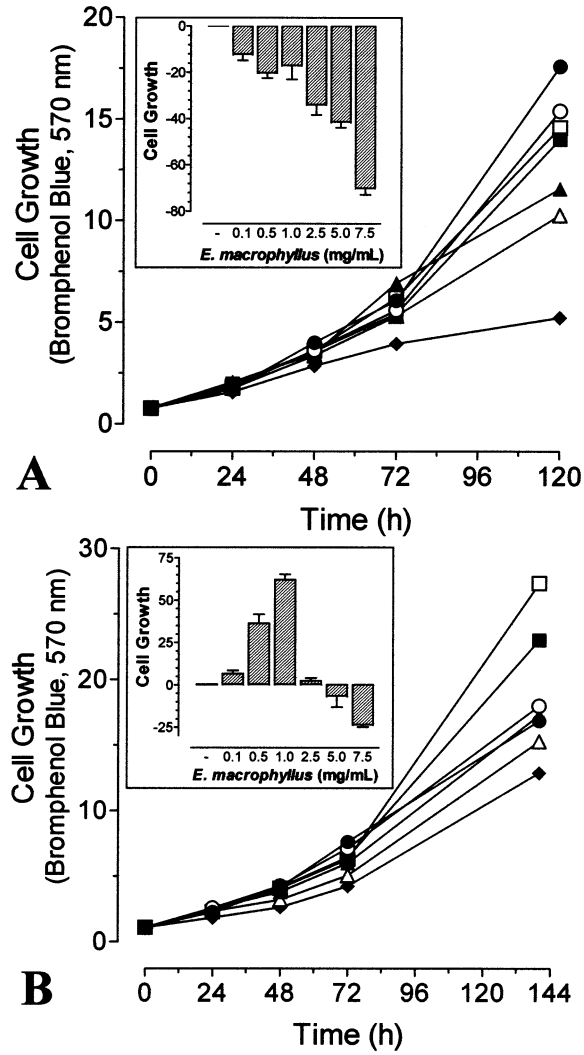

Fig. 1. Growth kinetics of HTC cells (A) and HEK cells (B), in the presence of a crude aqueous extract prepared from *E. macrophyllus* dried leaves. Exponentially growing cells in DMEM/10% FBS were grown for 5 to 6 days in the absence (●, control cells) or in the presence of different concentrations of *E. macrophyllus* crude aqueous extract (○, 0.1 mg/ml; ■, 0.5 mg/ml; □, 1.0 mg/ml; ▲, 2.5 mg/ml; △, 5.0 mg/ml, and ◆, 7.5 mg/ml), with a medium renew at 72 h. At the indicated times, cells were collected by fixation with 5% TCA and their number determined by staining with bromophenol blue, stain extracted with 10 mM Tris and absorbance read at 570 nm. Inserts A and B: data from last day of the growth kinetics of HTC cells (A) or HEK cells (B) treated with *E. macrophyllus* were compared to the last day of the growth kinetic of untreated cells (taken as 100%), to point out both stimulatory and inhibitory activities present in the *E. macrophyllus* aqueous extract.

not been observed on hepatoma cells (Fig. 1A). The putative modulation of kidney epithelial cell hypertrophy, however, is counteracted by a cell growth inhibitory activity additionally present in the *E. macrophyllus* extract (Fig. 1B).

3.3. Effects of a cumulative ingestion of E. macrophyllus on some growth parameters and plasma biochemistry

In vivo toxicological investigation was performed in male Swiss mice treated daily by free oral ingestion of an aqueous extract from *E. macrophyllus* dried leaves, either crude or lyophilized. After 6 weeks of treatment, animals were sacrificed and their body weight, organs (liver and kidneys) weight and markers of functional changes of liver and kidney were analyzed. Average daily intake of *E. macrophyllus* solution (ml/24 h/animal) was: control, 8.9 ± 0.3; group 1, 8.3 ± 0.2; group 2, 5.7 ± 0.2; group 3, 8.1 ± 0.2; and group 4, 7.0 ± 2.0. Based on these intakes, the following average daily exposure doses of the herb have been tested: group 1, 3.0 mg/kg; group 2, 23 mg/kg, group 3, 297 mg/kg (lyophilized extract), and group 4, 2200 mg/kg (crude extract). In comparison, the recommended daily dose to humans vary between 100 to 300 mg/kg, which is equivalent to 13 to 27 mg/kg of the lyophilized extract (dose 2).

Table 2 shows the results of the body and organ weights reached at the end of the experiment. Less net weight gain has been observed in all treated mice, particularly in groups 1 and 2, with a reduction in 47 and 42%, respectively, when compared to the net weight gain of control animals. This gross reduction in final body mass cannot be explained simply based on a diuretic effect of the herb, a presumed physiological action of *E. macrophyllus*. Since food intake was not measured in this study, we cannot rule out that the reported reduction of body weight was a consequence of anorexia. Impaired food absorption, or an effect on energy expenditure by body tissues, or both, are other mechanisms of action that can be involved. On the other hand, increasing the exposure dose by 10 times (group 3), induced a reduction of 5% only in the final body weight

gain. This effect has been also observed in group 4 (14% reduction), whose dose of crude extract was equivalent to the dose of the lyophilized extract given to group 3. Since there is an inverse relationship between *E. macrophyllus* intake and body weight lost, this effect can not be merely a consequence of a toxic effect of the herb. It might be due to the presence of pharmacological substances either active in very low doses and/or whose activities are counteracted by other substances present at smaller amounts.

No expressive changes were noticed among weights of liver and kidneys from animals treated with the lyophilized extract, in any dose tested (Table 2, groups 1 to 3). However, a reduction of 15% has been detected ($P < 0.05$) in the kidneys weight of group 4 (Table 2), showing again a particular effect on kidney cells. Urine sample analysis by electrophoresis did not exhibit any significant lost of plasma proteins, ruling out any suggestive harmful effect on glomerular permeability (data not shown). In addition, renal mark-

Table 2
Influence of cumulative ingestion of *E. macrophyllus* on body, liver and kidneys weight[a]

Treatment	Body weight (g)			Wet organs weight (g/100 g body weight)	
	Initial	Final	Net gain	Liver	Kidneys (both)
Control ($n = 6$)	32.3 ± 1.6	36.6 ± 1.7	4.3	5.33 ± 0.14	1.44 ± 0.06
Lyophilized extract:					
Group 1 (3 mg/kg ($n = 7$))	33.2 ± 1.9	35.5 ± 1.7	2.3	5.10 ± 0.16	1.33 ± 0.05
Group 2 (23 mg/kg ($n = 6$))	30.2 ± 1.7	32.7 ± 2.6	2.5	4.99 ± 0.14	1.32 ± 0.05
Group 3 (297 mg/kg ($n = 5$))	32.2 ± 0.7	36.3 ± 1.1	4.1	5.09 ± 0.18	1.35 ± 0.04
Crude extract:					
Group 4 (2200 mg/kg ($n = 5$))	30.7 ± 1.9	34.4 ± 3.3	3.7	5.01 ± 0.10	1.22 ± 0.04*

[a] Animals were treated for 6 weeks with *E. macrophyllus* aqueous extract added to the drinking water. Doses correspond to the average daily ingested dose of aqueous extract either lyophilized or crude. Control group received ordinary tap water. Data are expressed as mean ± standard error.

* $P < 0.05$ (student's t test).

Table 3
Plasma biochemical profile of Swiss mice after cumulative ingestion of *E. macrophyllus* extract in male Swiss mice[a]

Parameter	Control	*E. macrophyllus* aqueous extract			
		Lyophilized			Crude
		Group 1 (3 mg/kg)	Group 2 (23 mg/kg)	Group 3 (297 mg/kg)	Group 4 (2220 mg/kg)
Plasma chemistry					
Urea nitrogen (mg/dl)	50.1 ± 3.5	53.7 ± 2.7	45.3 ± 3.8	46.6 ± 5.7	45.2 ± 3.1
Creatinine (mg/dl)	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0.03	0.2 ± 0
Total protein (g/dl)	3.9 ± 0.2	4.1 ± 0.2	3.9 ± 0.2	3.8 ± 0.2	3.9 ± 0.1
Albumin (g/dl)	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	2.1 ± 0.2	2.2 ± 0.1
Globulins (g/dl)	1.9 ± 0.2	2.0 ± 0.1	1.9 ± 0.2	1.7 ± 0.1	1.7 ± 0.1
Enzyme (IU/l, 37°C)					
AST	482 ± 60	361 ± 25	594 ± 83	590 ± 101	356 ± 47
ALT	45 ± 5	43 ± 6	89 ± 14*	97 ± 34	53 ± 5
ALP	61 ± 7	68 ± 8	53 ± 14	61 ± 11	64 ± 4

[a] Control group received ordinary tap water. Data are expressed as mean ± standard error.

* $P < 0.05$ (student's t test).

ers of functionality like urea and creatinine plasma levels did not show any expressive alteration (Table 3). Likewise, no gross alterations were observed in plasma levels of total plasma proteins, albumin, and globulins (Table 3). As regards to plasma enzyme determinations, an increase in ALT has been detected in groups 2 ($P < 0.05$) and 3 (despite not statistically significant) (Table 3) without any concomitant alteration in the activity of AST and/or ALP. Since ALT is a marker of liver function, a modest increase in its plasmatic activity might indicate a subclinical hepatic intoxication without any deleterious consequence to the organism. On the other hand, no augmented ALT activity could be observed in animals of group 4. In summary, except for a significant reduction in kidney weight and in mass body gain, no other meaningful physiological alteration has been identified in the animals treated subchronically with *E. macrophyllus* aqueous extract.

3.4. Effect of subchronic ingestion of E. macrophyllus on DNA integrity of different tissues

To identify if *E. macrophyllus* leaves could contain substances potentially genotoxic to mammalian cells, DNA of liver, kidney, and blood cells of the treated animals was examined by the comet assay and the results obtained are shown in Fig. 2. When blood and liver cells were analyzed, no detectable DNA lesions have been observed in animals treated with the *E. macrophyllus* aqueous extract, either lyophilized or crude (Fig. 2A and B). Nevertheless, DNA evaluation of kidney cells indicated the presence of low to moderate frequency of lesions, particularly in animals treated with the highest dose of the herb (Fig. 2C, groups 3 and 4). In group 3 (lyophilized extract), two out of four animals evaluated indicated the presence of a high frequency of kidney cells with DNA lesions type 1 (42% (Fig. 2C, track 15) and 20% (Fig. 2C, track 16), respectively). Most of their kidney cells did not show any detectable DNA lesion (56 and 74%, respectively). However, the frequency of kidney cells from control animals with any detectable DNA lesion ranged from 80 to 92% (Fig. 2C, tracks 1 to 4). In group 4 (treated with crude extract), one out of four animals analyzed (Fig. 2C, track 20) showed a very high frequency of kidney cells with DNA lesion type 1 (50%) and DNA lesion type 2 (24%). Only 18% of the kidney cells of this particular animal did not show any detectable DNA lesion. Therefore, the highest tested dose of *E. macrophyllus* aqueous extract, either lyophilized (group 3) or crude (group 4), indicated the presence of substances potentially genotoxic to kidney cells, with crude extract showing a more drastic effect. Additional genotoxic evaluations, e.g. mammalian cell mutation test and in vivo chromosome aberration test, should be performed in order to clarify the genotoxic potential of this particular herb to mammalian organism. This is important in conditions of overdoses and long-term treatments, resulting in a cumulative buildup of the herb in the organism.

On the other hand, doses up to 23 mg/kg per day of the lyophilized *E. macrophyllus* aqueous extract, did not induce any detectable genotoxic effect on kidney cells (Fig. 2 C, tracks 5 to 12) or any other sign of toxicity to the organism. As this dose is equivalent to the daily dose recommended to humans on a weight basis, controlled ingestion of *E. macrophyllus* aqueous extract will not be harmful to human organism. This is also evidenced if the dosage of the herb given to humans and mice would be expressed per surface area (mg/cm^2) instead of mg per kg of body weight. In this case, the dosage would be approximately 10 times less in mice when compared to the equivalent dose ingested by humans.

In conclusion, *E. macrophyllus* aqueous extract seems to contain substances that specifically act on kidney cells. Preliminary chemical analysis of the lyophilized extract indicated that the same is quite rich in highly polar substances having as a common feature the presence of one or more phenolic hydroxy groups, e.g. polyphenols, phenolic acids, hydroxyflavonoids and hydroxystilbenes. No tannins or saponins could be detected (data not shown). Current studies are on the way to identify the referred phenolic substances.

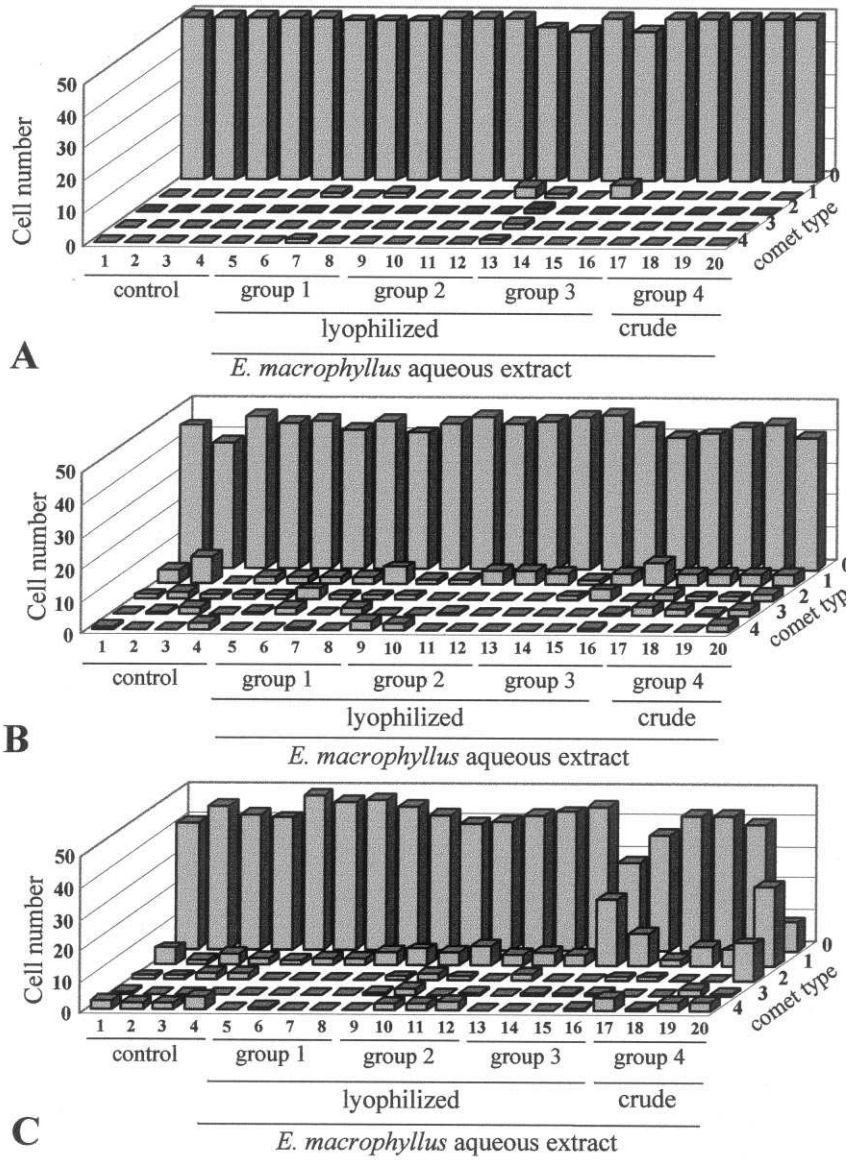

Fig. 2. Genotoxic potential determination on blood (A), liver (B) and kidney (C) cells after subchronic ingestion of *E. macrophyllus* aqueous extract, either lyophilized (groups 1, 2 and 3) or crude (group 4). Male Swiss mice were treated for 6 weeks with *E. macrophyllus* aqueous extract in drinking water after which their blood, kidney and liver cells were analyzed for the presence of DNA strand breaks by the comet assay. Each track represents investigation data from a single animal. Four animals were analyzed per treatment. Doses tested were: group 1, 3 mg/kg; group 2, 23 mg/kg; group 3, 297 mg/kg; and group 4, 2.2 g/kg.

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