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Safety assessment of aqueous extract from leaf *Smallanthus sonchifolius* and its main active lactone, *enhydrin*

Carolina Serra Barcellona [a], Wilfredo Marcelino Cabrera [a], Stella Maris Honoré [a], María Inés Mercado [b], Sara Serafina Sánchez [a,*], Susana Beatriz Genta [a]

[a] *Biological Research Institute (INSIBIO), National Council of Scientific and Technical Research (CONICET) and National University of Tucumán, Chacabuco 461, (T4000INI) San Miguel de Tucumán, Argentina*
[b] *Institute of Organic Chemistry, Biochemistry Chemistry and Pharmacy Faculty, National University of Tucumán, Ayacucho 491, (T4000INI) San Miguel de Tucumán, Argentina.*

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ABSTRACT

Ethnopharmacological Relevance: Leaves of *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson (yacon) have been used since pre-Columbian times in the Andean region to prepare medicinal herbal tea with beneficial health properties. However, there are still disagreements about the safe use. This work was carried out to evaluate the toxicity profile of both, 10% decoction of yacon leaves and their major active lactone, enhydrin.

Materials and methods: In vitro cytotoxicity assays were performed with Hep-G2, COS1, CHO-K1 and Vero cell lines using a test of metabolic competence based upon assessment of mitochondrial performance. *In vivo* toxicity study was performed in adult Wistar rats. In the acute oral toxicity each group of rats was orally given a single dose of 10% decoction or enhydrin. General condition, behavior and mortality were recorded for up to 14 days post treatment. In subchronic toxicity studies, both products were given orally for 90 days to rats. Body weight and food intakes were observed weekly. Hematological, clinical chemistry parameters and organ weight were determined in all animals at the end of the experimental period.

Results: Cell viability decreased in a concentration dependent fashion when cells were incubated with 2–200 μg of 10% decoction and 0.015–7.5 μg of enhydrin. In acute study in rats, there were no deaths or signs of toxicity observed after oral administration of single doses of 10% decoction or enhydrin at any dose level up to the highest dose tested (14.0 g/kg and 0.32 g/kg, respectively). In subchronic studies in rats, both products administered orally for 90 days at daily doses of 0.07, 0.14 and 0.28 g 10% decoction/kg and 0.4, 0.8 and 8.0 mg enhydrin/kg, did not caused haematological, biochemical and histological alterations.

Conclusions: The results presented in this paper lead us to the conclusion that the use of 10% decoction and enhydrin is safe in rat at doses in which it is demonstrated the hypoglycaemic effect.

1. Introduction

Yacon (*Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson) is a perennial herb native to South America that belongs to the family Compositae or Asteraceae. Yacon roots have a long history of use as part of the diet of the inhabitants of the Andes since pre-Columbian times (Grau and Rea, 1997) and yacon cultivation and consumption have expanded in recent decades to several Asian and European countries. With regard to the medicinal uses of the plant, some beneficial properties have been attributed to yacon leaves, which are used to prepare a medicinal tea (Aybar et al., 2001; Valentová and Ulrichová, 2003). In the past decade numerous studies have shown that the extracts of dried yacon leaves have a variety of pharmacological activities, including antimicrobial (Joung et al., 2010; Lin et al., 2003), anti-inflammatory (Hong et al., 2008), antioxidant and free radical scavenging properties (Valentová et al., 2003, 2005).

In a previous study, we demonstrated the hypoglycaemic effect of the water extract of *S. sonchifolius* leaves in normal and diabetic rats (Aybar et al., 2001). Interestingly, treatment of diabetic rats with aqueous extract of yacon leaves for 30 days improved the general condition, body weight and renal function of the animals. More recently, we found that the butanolic extract of yacon leaves showed a high effective hypoglycaemic activity in normoglycaemic, transiently hyperglycaemic and diabetic rats in a dose-dependent manner (Genta et al., 2010). On the basis of the

* Corresponding author. Tel.: +54 381 4107214; fax: +54 381 4247752x7004.
E-mail address: ssanchez@fbqf.unt.edu.ar (S.S. Sánchez).

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high polarity of this extract and the presence of caffeic and chlorogenic acid and their dicaffeoil quinic derivates, we may assume that these major constituents are active principles related to the hypoglycaemic effect of yacon leaves.

Further phytochemical investigations allowed the isolation and identification of a variety of compounds including melampolide-type sesquiterpene lactones (STLs) such as fluctuanin, uvedalin and enhydrin (Hong et al., 2008; Schorr and Da Costa, 2005). Enhydrin is the main lactone isolated from the leaves of yacon and, in recent years, studies have suggested that it has anti-diabetic properties, so much so that it was included in a patented anti-diabetic pharmaceutical formulation (Kawashima et al., 2001). In order to identify the metabolites with hypoglycaemic activity, in a previous work we isolated crystalline enhydrin from leaf extracts of *S. sonchifolius*. We found it to be an active compound that helped in the decrease of post-prandial blood glucose levels and that was useful in the treatment of diabetic Wistar rats (Genta et al., 2010).

The beneficial effects associated with the consumption of organic or aqueous extracts of yacon leaves for long periods might suggest that they have a high safety margin. Although some acute toxicity tests have been evaluated (Genta et al., 2010), studies on the toxicity of these extracts after prolonged periods of consumption need to be extensively reviewed.

Based on their results, Ogose et al. (2009) judged that the administration of yacon extracts to rats for two generations had no effects on either the reproductive functions or the development of the liveborn pups. In a recent work, de Oliveira et al. (2011) showed that prolonged oral administration (90 days) of extracts of *S. sonchifolius* was associated with kidney damage and attributed it to the presence of sesquiterpene lactones in the extract. However, the analysis of the results presented by these authors shows that the aqueous extract of yacon leaves triggers biochemical and histopathological changes when administered at high doses (100 mg/kg). This represents a dose six times higher than that used in folk medicine and is a question that requires further studies.

The toxic and medicinal properties of many plants have been shown to correlate with the biological activity of sesquiterpene lactones isolated from their extracts (Scmidt, 1999). In this regard, enhydrin is the main sesquiterpene lactone isolated from the leaves of yacon (Dou et al., 2010) and the determination of its potential toxic effects is an important issue to establish potential risks associated with its intake.

The degree of side effects or toxicity presented by extracts or compounds of medicinal plants depends on many complex factors. The effects of a single large dose of a toxic substance may not necessarily reflect the risks associated with the long-term low-level consumption commonly used in folk medicine. In addition, long-term studies are essential to determine a range of bioactivities to a no-observed-adverse-effect level (NOAEL) (Alexeeff et al., 2002).

Thus, this work was undertaken to evaluate the *in vitro* and *in vivo* toxicity of both the aqueous extract of *S. sonchifolius* leaves and enhydrin isolated from yacon leaves. It is an important issue in order to continue assessing their potential antidiabetic use.

2. Materials and methods

2.1. Plant material

Leaves of *S. sonchifolius* (Poepp & Endl.) H. Robinson (clone LIEY 97-1) used in this study were collected on February 2010 from plants cultivated in an experimental field belonging to the Regional Ecology Institute (IER), National University of Tucumán, located at Horco Molle, Yerba Buena, province of Tucumán, Argentina. Voucher specimens (LIL607173) are deposited in the herbarium of "Fundación Miguel Lillo", San Miguel de Tucumán, Argentina.

2.2. Preparation of the aqueous extract of S. sonchifolius

The plant material was carefully dried under air flow in an oven between 40 and 45 °C.

Aqueous extract of the plant was prepared by boiling 10 g dried leaves in 100 mL distilled water under reflux for 10 min. The decoction obtained (10%) was filtered, frozen at −20 °C and then lyophilized. The extract yielded 1.7 g of dry residue, which was stored at −20 °C until used. The appropriate amount of dry residue was dissolved in distilled water immediately before each experiment.

In the present work, we selected a 10% decoction based on its efficacy hypoglucemiant previously assayed in our laboratory (Aybar et al., 2001) and on the approximate dose used in traditional medicine (Grau and Rea, 1997). In addition, we tested different doses of 10% decoction in Wistar rats, being 0.14 g dry residue/kg a dose hypoglucemiant more effective.

2.3. Isolation and purification of enhydrin

To isolate preparative amounts of enhydrin, a procedure described by Genta et al. (2010) was used. The aim of this experimental procedure was to extract the contents of glandular trichomes (rich in STLs) of the leaf surface and from there to purify enhydrin.

2.4. Phytochemical analysis of the aqueous extract of S. sonchifolius

2.4.1. Infrared (IR) spectroscopy

The analysis of the 10% decoction was performed by IR spectroscopy. The samples were prepared in KBr tablets and the IR spectra were performed in a Perkin-Elmer 1600 FT-IR spectrophotometer.

2.4.2. Thin-layer chromatography (TLC) analysis

TLC analysis enabled identification of caffeic acid and chlorogenic acid (3-caffeoylquinic acid) by comparison with authentic samples using different solvents and detection systems. Merck aluminum sheets of Silica gel 60 F254 were used. For chlorogenic acid identification, plates were developed with *n*-butanol:acetic acid:water (10:1.75:8) or ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:27). For caffeic acid identification, plates were developed with *n*-hexane:ethyl acetate:acetic acid (4:6:0.15). Detection was performed by (i) fluorescence at 366 nm (Mineral Light Lamp, Model UV GL, multiband UV 254/366, UVP San Gabriel, USA), (ii) spraying with a 1% solution of 2-aminoethyldiphenylborinate in methanol and then with a 5% solution of polyethylene glycol in ethanol and (iii) spraying with a 1% solution of $FeCl_3$ in methanol.

2.4.3. HPLC analysis

For the HPLC analysis, a Gilson 322 HPLC (binary pump) with a Gilson UV/VIS-152 Detector, Rheodyne injector with 20 μL loop and Unipoint software was used. The column employed was a Grace Smart RP_{18} analytical column (5 μm; 4.6 mm × 250 mm) using two different solvent programs. Mobile phase I: solvent A (5% acetic acid–water solution); solvent B (methanol). Elution was achieved with the following linear gradient: 20 to 33.5% B in 60 min. Flow rate: 0.7 mL/min. UV detection was carried out at 326 nm; 0.01 sensitivity. Injection volume: 20 μL. Mobile phase II: solvent A (2% acetic acid–water solution); solvent B (2% acetic

acid–methanol solution). Elution was achieved with the following gradient: 15 to 40% B in 30 min; 40 to 75% B in 10 min and 75 to 85% B in 5 min at a flow rate of 0.7 mL/min. UV detection was carried out at 326 nm; 0.01 sensitivity. Injection volume: 20 μL. Identification was performed by comparison of retention times, co-injection with authentic samples and UV spectra. Caffeic acid and chlorogenic acid were obtained from Sigma Chemical Company. Standard solutions were prepared by dissolving 10 mg of either caffeic acid or chlorogenic acid in 100 mL of methanol (100 ppm, w/v solution). Reference samples of the three dicaffeoilquinic acids (DCQ), 3,4-DCQ, 3,5-DCQ and 4,5-DCQ were obtained from *Ilex paraguariensis* (Filip et al., 2001).

Additionally, a third solvent program was used. The binary mobile phase consisted of solvent A (0.5% acetic acid–water solution) and solvent B (0.5% acetic acid acetonitrile solution). Elution was achieved with the following linear gradient: 0 to 45% B in 30 min. Isocratic elution occurred with 45% B in 30 to 50 min, 45 to 100% B in 50 to 80 min. The flow rate was 1.3 mL/min. UV detection was carried out at 254 nm; 0.01 sensitivity. Injection volume: 20 μL. Identification was performed by comparison of retention times, co-injection with authentic samples and UV spectra. Authentic standards, enhydrin and uvedalin were obtained from *S. sonchifolius*.

2.4.4. Gas chromatographic analysis and mass spectra (GC–MS)

Dried 10% decoction of yacon leaves (50 mg) was resuspended in water (20 mL) and then extracted with 15 mL chloroform. The organic phase was recovered and solvent evaporation afforded 6.0 mg of residue. GC–MS analysis were recorded using a Hewlett-Packard 5973 selective mass detector coupled to a Hewlett-Packard 6890 Gas Chromatograph fitted with an Elite-5 MS Perkin-Elmer column (5% phenylmethylsiloxane, 30 m × 0.25 mm i.d.; 0.25 μm film thickness); ionization energy 70 eV. The following conditions were employed to analyze enhydrin: injector, GC–MS interphase, ion source and selective mass detector temperatures were maintained at 220 °C, 280 °C, 230 °C and 150 °C, respectively; injection size: 1.0 μL (split mode); carrier gas was Helium with a flow rate of 1.2 mL/min. The oven was programmed as follows: from 180 to 300 °C at 2 °C/min and then held at 300 °C for 10 min. The sample was injected as a 4% solution in methylene chloride. Enhydrin was characterized by: (a) comparison of its mass spectra with a commercial GC–mass spectra library (National Institute of Standards and Technology (1999). PC Version 1.7 of the NIST/EPA/NIH Mass Spectral Library. Perkin-Elmer Corp.: Norwalk, CT.) and with mass spectra reported in the literature (Hong et al., 2008); (b) co-injection with an authentic sample of enhydrin.

2.5. Study of toxicity in vitro

2.5.1. Cell culture

Hep-G2, COS1, CHO-K1 and Vero cell lines (ATCC, Manassas, VA, USA) were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% (*v*/*v*) FCS (fetal calf serum) (Gibco BRL, Gaithersburg, MD, USA) in 96-well microplates at 37 °C in a humidified atmosphere with 5% CO_2 in the air, as recommended by the ATCC.

2.5.2. Treatment of cell cultures

Cells were detached from culture flasks by trypsinization and suspended in the culture medium supplemented with 10% fetal bovine serum. An aliquot of suspended cells was taken to determine cell viability by trypan blue dye exclusion. Then the cells were seeded in 96-well culture plates at a density of approximately 1.5×10^4 cells/well. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air until confluence. The confluent cells were washed with Hank's balanced salt solution (HBSS). Then, the cells were incubated with test agents dissolved in supplemented culture medium containing 0.25% dimethyl sulfoxide (DMSO) for 48 h at 37 °C in 5% CO_2 (100 μL/well). The doses used of 10% decoction of *S. sonchifolius* were: 2, 10, 20, 100 and 200 μg of dry extract/mL and enhydrin doses were: 0.015, 0.07, 0.15, 0.75, 1.50 and 7.50 μg enhydrin/mL. After the treatment period, the cells were processed for cytotoxicity assays. Each assay was performed in triplicate. Negative and positive controls of cytotoxicity were performed by incubating the cells with DMEM and staurosporine, respectively. The results were expressed as the percentage of live cells.

2.5.3. Cytotoxicity assay

The MTT assay is a test of metabolic competence based upon assessment of mitochondrial performance. It is a colorimetric assay relying on the conversion of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Briefly, after the treatment, the medium was discarded and the microplates were washed with phosphate buffered saline. The cells were then incubated with MTT (0.5 mg/mL, 100 μL) in serum-free medium for 4 h at 37 °C to allow formation of crystals of formazan. Then, the medium was removed and 100 μL of glacial acetic acid was added to dissolve the formazan crystals. Absorbance was measured in an ELISA microtiter plate reader (BIORAD 680) at 540 nm. Cell viability was expressed as the percent viability of treated cells compared with the untreated control.

2.6. Study of toxicity in vivo

2.6.1. Animals

Adult male and female Wistar rats aged 8 to 12 weeks (weight 200 ± 20 g) were selected for all the experiments. They were obtained from the colony bred at the Department of Developmental Biology, INSIBIO (CONICET-UNT), Tucumán, Argentina.

The animals were acclimated for 7 days, during which period each animal was examined to confirm suitability for the study. Criteria for suitability were acceptable physical examination and body weight (b.w.), so that b.w. means for each group within a sex were comparable. At the beginning of the study, male rats weighed 200.0–220.0 g (mean = 217.0 g) and females 180.0–210.0 g (mean = 206.0 g).

The rats were housed in individual cages. The room was kept on a 12 h light/dark cycle. Temperature and humidity were maintained within specific ranges of 22 ± 2 °C and 60–70% relative humidity. Animals were given free access to a powdered certified rodent diet obtained from a commercial source (Standard Food – Asociación de Cooperativas Argentinas – SENASA N° 2706) and given tap water *ad libitum*. There were no known contaminants in food or water which were expected to interfere with the results of the study.

Throughout the experiments, all animals were maintained and handled according to international ethical guidelines for the care of laboratory animals (US Food and Drug Administration). The experimental protocol of toxicological studies was approved by the Committee on Bioethics in Research of the National University of Tucumán and all experiments have complied with the current laws of Argentina.

2.6.2. Experimental design

2.6.2.1. Acute oral toxicity study in rats. The acute oral toxicity of 10% decoction and enhydrin was evaluated in a single-dose study. Wistar rats were randomly divided into groups of six animals. Each group included three males and three females with a weight

of 200.0 ± 20.0 g that were orally given a single dose of 10% decoction or enhydrin. The doses tested were 3.5; 7.0 and 14.0 g dry extract/kg b.w. for 10% decoction, representing 25, 50 and 100 times the more effective hypoglycaemic dose, and 0.08, 0.16 and 0.32 g/kg b.w. for enhydrin, representing 100, 200 and 400 times the effective hypoglycaemic dose. The control group received only the vehicle (distilled water).

Rats were observed with respect to behaviour (reduction in locomotion, aggressiveness, reaction to stimuli as tail pinch, noise, social interactions), general condition, autonomic (piloerection, lacrimation, pupil size, respiratory pattern) and nervous central activity (posture, exploratory movements, stereotypes, presence of clonic or tonic movements) and mortality continuously for 1 h after treatment, then at 3.0 and 6.0 h post-dose on day 0 and twice daily (morning and afternoon) thereafter for 14 days. At this time, the animals were sacrificed under anaesthesia (i.p. ketamine, 150 mg/kg b.w.) and selected organs were separated and processed for histopathological examination.

2.6.2.2. Subchronic oral toxicity study of 10% decoction of S. sonchifolius leaves. The rats considered suitable for the study were distributed into four group of 10 animals each (5 of each sex): Group I received 10% decoction (0.07 g dry extract/kg b.w./day), Group II received 10% decoction (0.14 g dry extract/kg b.w./day), Group III received 10% decoction (0.28 g dry extract/kg b.w./day). Group IV received water and was used as control.

The appropriate amount of dry extracts was dissolved in distilled water immediately before use and was administrated orally once a day at 6:00 pm before eating with an intragastric tube. All the animals received the standard diet and water *ad libitum*. The experimental period was 90 days, a reasonable time period to establish a subchronic toxicity profile. (Food Standards Australia—New Zealand, 1998). Feed and water consumption and body weight were measured weekly during the experimental period. Clinical observations, behavioural profile (stereotypes, irritability and sedation), autonomic activity (salivation, piloerection and lacrimation, pupil size, breathing pattern, abdominal contortion, emesis and diarrhoea) and nervous activity (posture, exploratory movements, presence of clonic and/or tonic movements) were performed daily. Blood samples for haematology and clinical chemistry studies were obtained by amputation of the tail tip in fasting conditions every 30-day period and by cardiac puncture at the end of the experimental period. At this time, the animals were fasted overnight, but allowed access to water *ad libitum* and then sacrificed under anaesthesia (i.p. ketamine, 150 mg/kg b.w.). All the organs were carefully examined macroscopically *in situ* and the liver, kidney and entire gastrointestinal tract excised, weighed and fixed in 10% neutral formaldehyde for histopathological examinations.

2.6.2.3. Subchronic oral toxicity study of enhydrin. A similar experimental design to that of previous section was used to determine the sub-chronic toxicity of enhydrin at repeated doses. The rats were housed in cages and divided into four groups of ten animals each (5 of each sex): Group I received enhydrin 0.4 mg/kg b.w./day, Group II received enhydrin 0.8 mg/kg b.w./day, Group III received enhydrin 8.0 mg kg b.w./day. The dose of 0.8 mg/kg b.w. represents the effective hypoglycaemic dose determined in previous experiments (Genta et al., 2010). The other doses are half and 10 times the effective dose. Group IV received distilled water and was used as control.

The appropriate amount of enhydrin to achieve each dose was resuspended in distilled water. All groups were treated daily for a period of 90 days. The clinical and biochemical controls and the final studies were carried out in a similar manner to that in the above section.

2.6.3. Haematological and biochemical analysis

Blood for haematology studies was collected in tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). The automatic haematological analyzer CELL-DYN ® 3700 (Abbott) was used to measure the following parameters: erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin, platelet count, WBC total and differential count. Blood for clinical chemistry studies was collected in glass tubes without anticoagulant, allowed to clot for 1 h at room temperature and, after retraction of the clot and then centrifuged at 4000 rpm for 15 min to collect the serum. A fully automated cobas® 6000 analyzer (Roche) was used to measure the following parameters: glucose, total cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, blood urea nitrogen (BUN), total proteins and albumin.

2.6.4. Urinary analysis

Urine samples were collected in individual metabolic cages for a 12 h period at the end of the experimental period. This method allowed the obtainment of the urine without faecal contamination. Once collected the samples were examined for volume, specific gravity, pH, glucose, protein, ketones, bilirrubin, urobilinogen and blood pigments using test strips (Bayer Diagnostic Division Argentina SA). Urine sediment was examined in a light microscope (NIKON fluophot) after urine centrifugation at 1000 rpm for 10 min.

2.6.5. Histopathological examination

Liver, kidneys and gastrointestinal tract were fixed in 10% neutral formaldehyde in PBS pH 7 for histologicalpathological study. After completion of fixation, the samples were dehydrated in an alcohol series, cleared in xylene and embedded in Paraplast. Blocks were cut at a microtome setting of 5–7 μm, mounted on glass slides previously treated with HistoGrip (Zimed Lab, Inc. USA), stained with haematoxylin-eosin, examined and photographed using a light photomicroscope (Nikon, digital camera, 2 MPx).

2.7. Statistical analysis of data

The data of the *in vitro* effects were analyzed by one-way analysis of variance (ANOVA) using SPSS software version 12.0. p value < 0.05 was considered statistically significant.

The results from three independent *in vivo* experiments were presented as mean ± SD. The significance of differences was evaluated using the paired Student's t-test. When more than one group was compared with one control, significance was evaluated by ANOVA. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Phytochemical analysis of the aqueous extract (10% decoction) of S. sonchifolius leaves

An IR spectroscopy analysis was performed as a first approach towards the determination of the active principle(s) of 10% decoction of the yacon leaves. Absorption corresponding to γ-lactone carbonyl (1755–1780 cm^{-1}) was observed in the IR spectrum of the 10% decoction, indicating that SLTs are present in this extract. The IR spectrum (KBr) of the 10% decoction displayed strong absorptions at 3300 cm^{-1} (ascribable to –O–H hydrogen bounding, polymeric association) and at 1600 cm^{-1} (alkenyl C=C stretch), indicating that phenolic acids are present in this extract.

Caffeic acid and chlorogenic acid were readily detected in the 10% decoction by TLC using authentic samples as reference and

three different detection systems. Also, all the three isomeric dicaffeoylquinic acids, *i.e.*, 3,4-DCQ, 3,5-DCQ and 4,5-DCQ acids were found to be major constituents of the decoction.

HPLC analyses of 10% decoction using two solvent programs (see Section 2.4.3) are shown in Fig. 1A shows HPLC chromatograms registered at 326 nm (Mobile phase I) with peaks corresponding to Chlorogenic acid and Caffeic acid. In Fig. 1B, the chromatograms using the Mobile phase II showed peaks of 3,4-DCQ, 3,5-DCQ and 4,5-DCQ acids (detection UV at 326 nm). The chromatogram registered at 254 nm (Fig. 2A) using a third solvent program demonstrates that the 10% decoction of yacon leaves has polar compounds and many peaks with low retention times. It also showed a single peak (3) with intermediate retention times and therefore of intermediate polarity, likely an STL. The presence of enhydrin or uvedadin in the 10% decoction was investigated by co-injection with authentic samples (Fig. 2B) where the comparison of the retention times indicated that peak 3 is not enhydrin or uvedalin.

To confirm or exclude the presence of enhydrin in the decoction of yacon leaves, a rapid and sensitive GC–MS method for analysis of STL in the 10% decoction was carried out. GC–MS analysis allows the identification of enhydrin as a minor component in the decoction.

3.2. Toxicity experiments in cell cultures

In the present study, we used four different cell lines to evaluate the potential toxicity of the 10% decoction of *S. sonchifolius* leaves and enhydrin, Hep-G2, COS1, CHO-K1 and Vero cell lines. Because of their high degree of morphological and functional differentiation *in vitro*, these cell lines are a suitable model to study the toxicity of xenobiotics, and are a useful tool to estimate starting doses for *in vivo* toxicity studies.

Cell viability decreased with increasing concentrations of the 10% decoction. The concentration at which cell viability was inhibited to 50% of control values (IC50) was 100 μg/mL of the 10% decoction for the Hep-G2 and CHO-K1 cell lines, 175 μg/mL of 10% decoction for the COS1 cell line and 125 μg/mL of 10% decoction for the Vero cell line. The IC50 values for enhydrin were 0.15, 0.75, 1.75 and 0.75 μg/mL for Hep-G2, CHO-K1, COS1 and Vero cell lines, respectively. The results indicate that COS1 cells, a cell line derived from kidney cells of monkey, were the most resistant to the treatment with both decoction and pure enhydrin.

3.3. Studies of toxicity in vivo

3.3.1. Acute oral toxicity studies

A wide range of doses of 10% decoction and enhydrin were assayed to evaluate acute toxic effect in healthy adult rats. No deaths or toxic effects (changes in behaviour or posture, presence of convulsions, occurrence of secretions, gastrointestinal symptoms such as diarrhoea or constipation, presence of blood in faeces) were observed at any of the doses tested. The animals did not lost weight and the microscopic examinations of selected organs (liver, kidney, gastrointestinal tract) also indicated that there was no evidence of toxicity at any dose level up to the highest dose tested of the 10% decoction (14.0 g dry extract/kg b.w., 100 times the effective hypoglycaemic dose) and enhydrin (0.32 g enhydrin/kg b.w., 400 times the effective hypoglycaemic dose). These doses represent the no-observed-adverse-effect level (NOAEL), indicating that the safety margin of this extract/active principle is high.

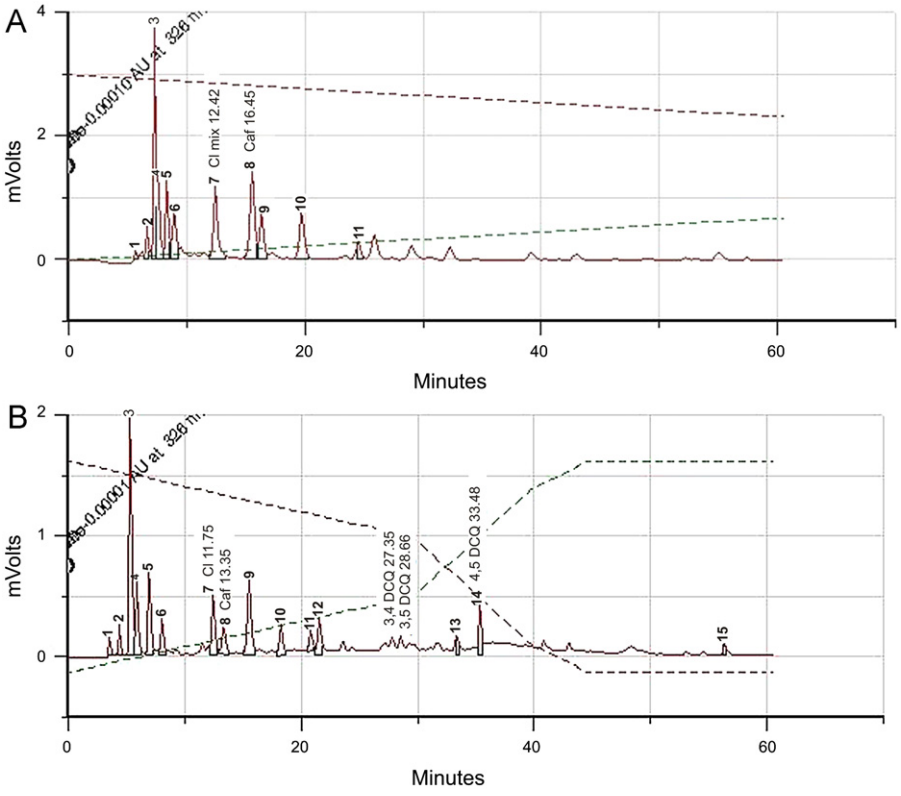

Fig. 1. HPLC chromatogram of 10% decoction from yacon leaves. Grace Smart RP18 analytical column ((5 μm; 4.6 mm × 250 mm). (A) Mobile phase I: solvent (a), 5% acetic acid-water solution; solvent (b), methanol. Linear gradient: 20 to 33.5% (b) in 60 min. Flow rate: 0.7 mL/min. (B) Mobile phase II: solvent (a), 2% acetic acid–water solution; solvent (b), 2% acetic acid–methanol solution. Gradient: 15–40% (b) in 30 min; 40–75% (b) in 10 min and 75–85% (b) in 5 min. Flow rate of 0.7 mL/min. UV detection: 326 nm. Caf: caffeic acid, Cl: chlorogenic acid, DCQ: dicaffeoilquinic acid.

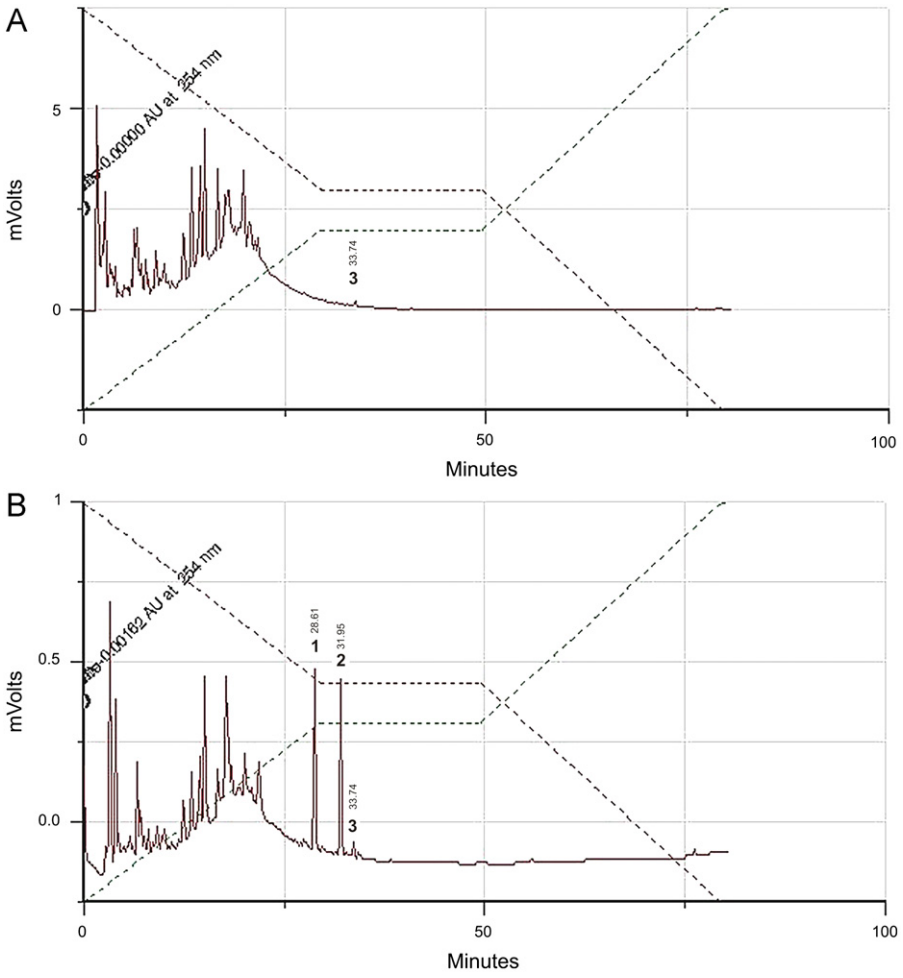

Fig. 2. HPLC chromatogram of 10% decoction from yacon leaves. Grace Smart RP18 analytical column ((5 μm; 4.6 mm × 250 mm). (A) Mobile phase: solvent (a), 0.5% acetic acid-water solution; solvent (b), 0.5% acetic acid–acetonitrile solution. Gradient: 0–45% (b) in 30 min, isocratic elution with 45% (b) in 30–45 min, 45–100% (b) in 50–80 min. Flow rate: 1.3 mL/min. UV detection: 254 nm. (B) Chromatogram of 10% decoction coinjected with enhydrin (1) and uvedalin (2) obtained from *S. sonchifolius* leaves. Column and conditions were identical to (A).

Table 1
Effect of the subchronic oral administration of 10% decoction of *Smallanthus sonchifolius* leaves on body weight.

Animal groups	Initial weight (g)	Final weight (g)	Weight gained (g)
Group I (0.07 g/kg/day)	208.3 ± 3.1	312.5 ± 11.5	104.2 ± 8.4*
Group II (0.14 g/kg/day)	205.9 ± 4.4	305.2 ± 13.3	99.3 ± 8.9*
Group III (0.28 g/kg/day)	210.0 ± 7.3	310.0 ± 8.2	100.0 ± 0.9*
Group IV (control)	199.3 ± 10.0	317.7 ± 7.5	118.4 ± 2.5

Data are mean ± SD. $n = 10$ animals (5 males and 5 females) per group.

* $p < 0.05$ was considered statistically significant compared with the control group (Group IV).

3.3.2. Subchronic toxicity studies

3.3.2.1. Effect of subchronic oral administration of 10% decoction of yacon leaves. In the present study, three doses of 10% decoction of *S. sonchifolius* leaves were administered orally, 0.07, 0.14 and 0.28 g dry extract/kg b.w./day, being 0.14 g/kg b.w./day a dose hypoglucemiant more effective. During the experimental period no signs of toxic effects or mortality were evident, nor were significant clinically relevant changes observed in general behaviour or physiological activities.

The animals that received different doses of the decoction showed no variation in daily standard rodent diet consumption compared with the untreated control group. Food intake was 58.1 ± 0.2, 58.0 ± 0.4, 57.8 ± 0.2 and 57.9 ± 0.1 g/kg b.w./day for group I, II, III and IV (control), respectively. No differences were observed in the volume of water ingested throughout the experimental period.

Changes in body weight of treated and control animals are shown in Table 1. There were no significant differences in initial and final weight of treated animals compared to controls. The weight gain of rats treated with any of the doses of the 10% decoction was lower, although all groups of treated animals gained weight with time.

The haematology parameters measured after 90 day administration of different doses of 10% decoction remained within physiological ranges, without significant differences from the control group. Treatment for 90 days did not cause any statistically significant changes in serum glucose, total cholesterol, triglycerides, creatinine, urea, total proteins or albumin or in the activity of the

marker enzymes of liver injury (AST and ALT). Additionally, no significant changes in haematology or clinical biochemistry parameters were noted at 30 and 60 day of treatment with the 10% decoction

Urine analysis was carried out at the end of the experimental period (90 days), before euthanasia. No significant differences between rats treated with the 10% decoction and untreated control rats were observed in urinary parameters analysed. No abnormalities were detected in the excretion of proteins or glucose, as well as in urine volume and pH, indicating that the renal function was normal.

Necropsy and histological examination of selected organs were performed on day 90 in control and treated groups. Macroscopic observation *in situ* of liver, kidney, entire gastrointestinal tract, brain, lungs, heart, spleen, testes, prostate gland, uterus and ovaries did not show any changes due to the treatment with different doses of the 10% decoction. No significant treatment-related alterations were observed either in absolute organ weight or when these weights were expressed in relation to body weight.

Histopathological examination of selected organs (liver, kidney, gastrointestinal tract, brain, lungs, heart, spleen, testes, prostate gland, uterus and ovaries) did not reveal any microscopic lesion related to the treatment with the 10% decoction. Thus, we observed a normal histoarchitecture of the organs, comparable to that of the untreated control group.

3.3.2.2. Effect of subchronic oral administration of enhydrin. In this experimental design were assayed three doses of enhydrin purified from leaves of *S. sonchifolius*: 0.4, 0.8 and 8.0 mg pure enhydrin/kg b.w./day. The dose of 0.8 mg/kg b.w./day represents the effective hypoglycaemic dose determined in a previous work (Genta et al., 2010). The other doses are half and ten times the effective dose.

There were no deaths or signs of toxicity observed after the daily oral administration of enhydrin at any dose level up to the highest dose tested. Throughout the experimental period, no significant differences between control and treated groups were recorded in body weights. Table 2 shows the effect of enhydrin on changes in body weight. All groups of treated animals gained weight with time, the weight gain being higher in groups I and III compared with the control group.

Treatment with different doses of enhydin produced no significant changes in the daily consumption of food or water.

Subchronic oral administration of enhydrin (daily for a 90 day period) did not cause any significant alterations in the final haematological and serum biochemical parameters measured, compared with the control group. Additionally, no significant changes were noted on days 30 and 60 of treatment. The urinary parameters determined remained within physiological values after a treatment period of 90 days.

Similarly to the 10% decoction, oral administration of enhydrin for a period of 90 days did not produce any macroscopic abnormalities in the organs of treated rats. Microscopic evaluation of these organs showed no tissue injury (lesions) associated with the treatment such as cell degeneration, necrosis or inflammatory reactions No treatment-related alterations were observed in the absolute weight of liver, kidney or gastrointestinal tract or when these weights were expressed in relation to body weight.

4. Discussion

The efficacy and safety of extracts or active compounds of medicinal plants must be studied thoroughly to maximize their therapeutic benefits. In popular medicine, dried *S. sonchifolius* (yacon) leaves are used in the preparation of infusions or decoctions to treat hyperglycaemic disorders in diabetes mellitus (Grau and Rea, 1997; Valentová and Ulrichová, 2003). In fact, several phytochemical and pharmacological studies (Aybar et al., 2001; Genta et al., 2010; Valentová et al., 2005) of yacon leaves have been conducted in the recent decades. Although there is sufficient evidence of their potential activity in the treatment of hyperglycaemia and its complications, there is still disagreement about their safe use and the appropriate therapeutic dose (de Oliveira et al., 2011; Fernandes et al., 2005; Genta et al., 2010; Siriwan et al., 2011). In the current study, we demonstrated that both a 10% decoction of yacon leaves and enhydrin have a safety margin in Wistar rats at doses in which was demonstrated hypoglycaemic effects.

Before the *in vivo* preclinical toxicity test, *in vitro* tests are useful to assess toxicity in a preliminary way. The present work was carried out using four different cell lines, which represent different cellular origins. The selection of cell lines was based on the main target organs to potential adverse or toxic effects of the extracts or pure compound under investigation. Hep-G2 cells possess a liver-like enzyme pattern and are used as a model system for studies of liver toxicity of xenobiotics (Westerink and Schoonen, 2007). The COS1 is an monkey kidney fibroblast-like cell line and the CHO-K1 cell line was derived as a subclone from a parental CHO cell line initiated from the biopsy of an ovary of adult Chinese hamster. The Vero is a normal epithelial cell line and was initiated from the kidney of an adult monkey. In our *in vitro* assessment of cytotoxicity with MTT assay, 10% decoction and enhydrin showed a concentration-dependent decrease in mitochondrial function and consequently in cell viability. COS1 cells were the most resistant to the treatment with both decoction or enhydrin, and the normal epithelial Vero cells showed intermediate values of IC50, very similar to epithelial-like CHO-K1 cell line. It is known that the effects of an oral dose are subject to systemic bioavailability and hepatic metabolism, pharmacokinetic processes that are absent in a cell culture model (Singh, 2006). Therefore, the *in vitro* studies presented here represent important alternatives to complement animal studies *in vivo* and were used as an initial survey to evaluate potentially toxic doses of yacon leaves.

In traditional medicine, the decoction dose of yacon leaves administered to patients to achieve a blood glucose lowering effect is difficult to determine accurately and so is the dose at

Table 2
Effect of the subchronic oral administration of enhydrin on body weight.

Animal groups	Initial weight (g)	Final weight (g)	Weight gained (g)
Group I (0.4 mg/kg/day)	191.9 ± 4.7	273.5 ± 6.1	81.6 ± 1.4*
Group II (0.8 mg/kg/day)	196.3 ± 3.3	267.0 ± 5.9	70.7 ± 2.6
Group III (8.0 mg/kg/day)	200.1 ± 5.1	277.1 ± 5.8	77.0 ± 0.7*
Group IV (control)	202.0 ± 4.3	275.3 ± 4.2	73.3 ± 0.1

Data are mean ± SD. $n=10$ animals (5 males and 5 females) per group.

* $p < 0.05$ was considered statistically significant compared with the control group (Group IV).

which toxic effects may occur. Thus, the present study was performed to evaluate the complete toxicity profile of both the 10% decoction and enhydrin in rats.

In the acute toxicity experiments, a specific range of doses was selected and evaluated. The doses of the 10% decoction were 25, 50 and 100 times the effective hypoglycaemic dose and 100, 200 and 400 times the effective hypoglycaemic dose of enhydrin. The rats were controlled closely up to 14 days after administration and no signs of toxicity or deaths were recorded. The lack of toxic effects suggested that the LD50 of the 10% decoction and of enhydrin would be above 14.0 and 0.32 g/kg b.w., respectively, which are significantly higher than the effective hypoglycaemic doses. The result of the acute studies was used to set doses for longer term experiments. In fact, we analyzed the subchronic oral toxicity of a 10% decoction and enhydrin in a wide range of doses but never as high as the LD50, as was suggested by Tamborini et al. (1990).

Despite the traditional use of decoction yacon leaves, in the literature there are few studies that evaluate the toxic potential of both yacon leaves extract and pure compounds, and these are contradictory (de Oliveira et al., 2011; Fernandes et al., 2005; Genta et al., 2010; Ogose et al., 2009; Siriwan et al., 2011). Thus, the present study was performed to evaluate the subchronic toxicity profile of both the 10% decoction and pure enhydrin in rats. The results show that the 10% decoction orally administered is non toxic, at least up to the maximum level assayed (0.28 g/kg b.w./day). Similarly, isolated enhydrin has no toxic effects at a dose range of 0.4 to 8.0 mg/kg b.w./day. These values represent the non-observed-adverse-effects level (NOAEL), which include the effective hypoglycaemic dose of the extract and the lactone. Moreover, all treated groups gained body weight throughout the experimental period and no significant alterations were recorded in absolute or relative organ weight. These findings would support the safe use of yacon leaves, taking into account that, after an exposure to toxic compounds, there will be a reduction in body weight gain as well as in organs weight (Rosidah et al., 2009; Teo et al., 2002).

It is known that the hematopoietic system is sensitive to toxic substances, serving as a very important index of toxicity (Fliedner et al., 1990). Based on the haematological parameters analyzed, we concluded that both, 10% decoction and enhydrin were not toxic at the doses tested and did not interfere with the production of circulating red blood cells, white blood cells or platelets. For the other hand, changes in serum levels of AST or ALT enzymes occur when there is hepatocellular injury (Goodman, 2002). Necrosis of the liver cells leads to a significant rise in these enzymes in the blood and can provide a quantitative evaluation of the degree of damage to the organ. In the present study, the lack of this effect strongly indicates that yacon leaves compounds are non toxic for hepatocytes.

Drug-induced kidney injury is a common adverse effect. Increased levels of creatinine and urea in blood or microalbuminuria are indicators of kidney injury (Paller, 1990). Under our experimental conditions, treated rats showed no alterations in renal parameters, suggesting that neither the 10% decoction nor enhydrin at the doses assayed induce kidney damage. In addition, recently we demonstrated that 10% decoction is a protective agent against renal damage in diabetic nephropathy (Honoré et al., 2012), supporting the notion that the yacon leaves extract lacks the toxic effect on the kidney.

In a recent work, de Oliveira et al. (2011) observed that the prolonged oral administration of a 2% yacon infusion (dose, 100 mg/kg b.w.) and a leaf-rinse extract which is rich in sesquiterpenic lactones (dose, 100 mg/kg b.w.) resulted in renal toxicity. It should be noted that the dose of the 2% infusion that caused adverse effects is 5.6 times higher than the effective hypoglycaemic dose (18 mg dry extract/kg b.w.) determined previously in our laboratory (Aybar et al., 2001). Moreover, the authors attributed the toxic effect to the presence of sesquiterpene lactones in the extract. However, did not determine the exact amount of the lactone enhydrin in the leaf-rinse extract. Indeed, we believe that adverse effects may increase progressively with increasing doses.

In summary, the results presented in the present paper lead us to the important conclusion that the use of a 10% decoction and isolated enhydrin from yacon leaves is safe in rat at doses in which the hypoglycaemic effect is demonstrated. This finding supports the use of yacon leaves in traditional medicine.

5. Conclusions

The results presented in this paper lead us to the conclusion that the use of 10% decoction and enhydrin is safe in rat at doses in which it is demonstrated the hypoglycaemic effect.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2012.09.021.

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