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Polymatin A from *Smallanthus macroscyphus* leaves: A safe and promising antidiabetic compound

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Abstract: *Smallanthus macroscyphus* is an herb native to South America whose leaves are a source of antidiabetic compounds, although complete information about their safe use is not available yet. This study was developed to evaluate the toxicity profile of both 10% decoction and the sesquiterpene lactone polymatin A from *S. macroscyphus* leaves through *in vitro* cytotoxicity assays and *in vivo* subchronic oral toxicity. Cell viability of Hep-G2, COS1, CHO-K1 and Vero cell lines decreased in a concentration-dependent manner when cells were incubated with 0.4–200 μg ml^{-1} of dry extract or 0.12–60 μg ml^{-1} of polymatin A. In subchronic studies, decoction was orally administered to Wistar rats for 90 days at daily doses of 70, 140 and 280 mg kg^{-1} of dry extract, whereas polymatin A was administered in the same way at doses of 7, 14 and 28 mg kg^{-1}. No toxicity signs or deaths were observed. There were no changes in the behavior, body or organ weights, hematological, biochemical or urine parameters of the rats. No histopathological lesions were observed in the examined organs. The results indicate that the 10% decoction and polymatin A from *S. macroscyphus* leaves may be considered as non-toxic substances at a wide range of doses, including the effective hypoglycemic dose. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: *Smallanthus macroscyphus* leaves; polymatin A; *in vitro* toxicity; subchronic toxicity

Introduction

Diabetes mellitus is a group of chronic metabolic disorders with a high and growing global incidence (Imperatore *et al.*, 2012). It is a disease characterized by hyperglycemia whose main treatment is to keep blood glucose levels of patients within normal levels to reduce clinical complications and improve the quality of life of people with diabetes. To achieve this objective, in the last years considerable attention has been directed toward the identification of plants and their chemical compounds with antidiabetic ability that may be used for human consumption (Noor *et al.*, 2008; Patel *et al.*, 2012).

Smallanthus macroscyphus (Baker ex Martius) A. Grau (Heliantheae, Asteraceae) is a wild species native to northwestern Argentina and southern Bolivia where it is known under the common name “wild yacon” (Grau & Rea, 1997). In a recent study, we demonstrated for the first time that the 10% decoction of *S. macroscyphus* leaves has significant hypoglycemic activity and a relevant antidiabetic potential in an experimental model of diabetes in rats (Serra-Barcellona *et al.*, 2014). Moreover, polymatin A, the major melampolide-type sesquiterpene lactone (STLs), present in *S. macroscyphus* leaves, exerts an effective inhibition of postprandial blood glucose peak and a hypoglycemic activity in diabetic animals (Serra-Barcellona *et al.*, 2014).

While over the last decades, numerous lactones from many plants have acquired therapeutic relevance as single components for the local treatment of inflammation and for cancer therapy (De Ford *et al.*, 2015; Merfort, 2011). Other studies have shown that certain lactones have toxic properties (Heilmann *et al.*, 2001; Schmidt, 1999). Most of the observed biological/toxic effects have been attributed to the capability of these compounds to deactivate enzymes and other essential proteins by the formation of covalent bonds with free cysteine sulfhydryl groups in such polypeptides (Schmidt, 1999).

Importantly, knowledge on the safety use of STL Polymatin A, from *S. macroscyphus* leaves is essential to develop new pharmacologically active agents. Thus, the aim of this work was to determine the toxicity profile of *S. macroscyphus* leaf extract and particularly, of the pure crystalline isolated polymatin A by assessing *in vitro* toxicity tests and *in vivo* subchronic oral toxicity studies on male and female albino Wistar rats.

Materials and methods

Plant material

Leaves from *S. macroscyphus* (Baker ex Martius) Grau were collected in March 2014 from an experimental field (Regional Ecology Institute, National University of Tucuman, located at Horco Molle, Yerba Buena, province of Tucuman, Argentina, 26°47′S, 65°19′W, 547 m.a.s.l. A voucher specimen (LIL607375) is deposited in the herbarium of the “Fundacion Miguel Lillo,” San Miguel de Tucuman, Argentina.

Preparation of the aqueous extract of *S. macroscyphus* leaves. Phytochemical analysis

Fresh plant material (leaves) was carefully dried under an airflow in an oven between 40 and 45 °C and then ground to a powder. The

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aqueous extract (decoction) was prepared by boiling 10 g dried powder in 100 ml distilled water under reflux for 10 min. The decoction obtained (10%) was filtered, frozen at –20 °C and then lyophilized. The yield in dry residue was 1.8 g (18%, w/w), which was stored at –20 °C until used. The appropriate amount of dry residue was dissolved in distilled water immediately before each administration. In the present work, a 10% decoction was selected based on the previous study of the hypoglycemic efficacy of *S. macroscyphus* leaves (Serra-Barcellona *et al.*, 2014).

The dry lyophilized residue from the 10% decoction was analyzed by infrared spectroscopy, thin-layer chromatography, high-performance liquid chromatography (HPLC) and gas chromatography coupled to a mass detector (GC-MS) as was previously described by Serra-Barcellona *et al.* (2014).

Isolation and purification of polymatin A

Polymatin A was obtained from glandular trichomes of the leaf surface of *S. macroscyphus* as was described previously (Serra-Barcellona *et al.*, 2014). After the last step, recrystallization afforded 2.89 g of crystalline polymatin A, melting point 121–122 °C, which corresponds to a yield of 0.71% (w/w of dry leaf). GC-MS indicated a purity of 97.4% of polymatin A.

Animals

Adult male and female Wistar rats aged 8–12 weeks were obtained from the colony bred at the Department of Developmental Biology, INSIBIO (CONICET-UNT) (Tucuman, Argentina). The animals were acclimated for 7 days before the experiments. At the beginning of the study, the males weighed 200–220 g while the females weighed 180–210 g. All specimens were in good health.

The animals were kept in an environmentally controlled room (12 h dark/light cycle with lights on from 07:00 to 19:00 h, air changes, room temperature at 22 ± 2 °C and relative humidity of 60–70%).

The rats were given free access to a powdered certified rodent diet obtained from a commercial source (Standard Food-Asociacion de Cooperativas Argentinas, SENASA no. 2706) and given tap water *ad libitum*. There were no known contaminants in food or water that might 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 was approved by the Committee for Care and Use of Laboratory Animals of the National University of Tucuman and all experiments complied with the current laws of Argentina (Ethical Framework of Reference for Biomedical Research in laboratory animals, Resol. D no. 1047 anexo II, 2005).

In vivo antidiabetic activity

Diabetes was induced in overnight-fasted male rats (n = 20) by a single intraperitoneal injection of 45 mg kg^{-1} freshly prepared streptozotocin (STZ; Sigma Chemical Company, St. Louis, MO, USA) in 10 mM sodium citrate buffer, pH 4.5 (Genta *et al.*, 2010). Rats with blood glucose more than 350 mg dl^{-1}, 48 h after injection were included in the study. STZ diabetic rats were further divided into four groups (five animals in each group), and each group was treated with vehicle (distilled water, 1 ml); 10% decoction (140 mg kg^{-1} day^{-1}); polymatin A (14 mg kg^{-1} day^{-1}) or the antidiabetic reference drug glimepiride (5 mg kg^{-1} day^{-1}) for 4 weeks as described earlier (Serra-Barcellona *et al.*, 2014). Animals were regularly observed for their general behavior and blood glucose levels were determined weekly with an Accu-chekR Active (GnbHD-68298; Roche Diagnostics, Mannheim, Germany) to glycemic control. Changes in body weight were also measured.

In vitro cytotoxicity validation study

Cell culture. The *in vitro* cytotoxicity tests were carried out using the following cell lines: Hep-G2, COS1, CHO-K1 and Vero cell lines (ATCC, Manassas, VA, USA). These cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine 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.

Treatment of cell cultures. Cells were detached from culture flasks by trypsinization and suspended in the culture medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA). 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 per 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. Then, the cells were incubated with test agents dissolved in supplemented culture medium containing 0.25% dimethyl sulfoxide for 48 h at 37 °C in 5% CO_2 (100 μl per well). The doses of the 10% decoction of *S. macroscyphus* used were 0.4, 4, 40, 100 and 200 μg ml^{-1} of dry extract, while polymatin A doses were 0.12, 1.2, 12, 30 and 60 μg ml^{-1}. Each assay was performed in triplicate. Negative and positive cytotoxicity controls were performed by incubating the cells with Dulbecco's modified Eagle's medium and staurosporine, respectively. The results were expressed as the percentage of live cells.

Cytotoxicity assay

After the treatment period (48 h), the cells were processed for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after 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^{-1}, 100 μl) in serum-free medium for 4 h at 37 °C to allow the formation of formazan crystals. Then, the medium was removed and 100 μl of glacial acetic acid was added to dissolve the crystals. Absorbance was measured in an enzyme-linked immunosorbent assay microtiter plate reader Bio-Rad 680 (Bio-Rad Laboratories, Inc., California, USA) at 540 nm. Cell viability was expressed as percentage viability of treated cells compared with the untreated control.

In vivo toxicity studies

Subchronic oral toxicity study of 10% decoction of S. macroscyphus *leaves.* Normal rats considered suitable for the study were randomly distributed into four groups of 10 animals each (five of each sex): group I received 10% decoction in a dose of 70 mg kg^{-1} day^{-1}; group II received 10% decoction in a dose of 140 mg kg^{-1} day^{-1}; group III received 10% decoction in a dose of 280 mg kg^{-1} day^{-1}; and group IV received water and was used as a control. The dose range selected was previously determined (Serra-Barcellona *et al.*, 2014).

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The appropriate amount of dry extract was dissolved in distilled water immediately before use and was administered orally once a day at 18:00 h before feeding with an intragastric tube.

All the animals received the standard diet and water *ad libitum*. The experimental period was 90 days, a reasonable time to establish a subchronic toxicity profile according to the US Food and Drug Administration guidance. Gross appearance of animals, clinical observations, behavioral profile (stereotypes, irritability and sedation), autonomic activity (salivation, piloerection and lacrimation, pupil size, breathing pattern, abdominal contortion, emesis and diarrhea) and nervous activity (posture, exploratory movements, presence of clonic and/or tonic movements) were observed daily. Feed and water intake and body weight were measured once a week.

Blood samples for hematological and clinical biochemistry studies were obtained by amputation of the tail tip in fasting conditions every 30 days and by cardiac puncture at the end of the experimental period. Hematology parameters, including red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, total and differential white blood cell counts were measured with an automatic hematological analyzer CELLDYN® 3700 Abbott (Abbott Laboratories, Buenos Aires, Argentine). Serum biochemical parameters including glucose, total cholesterol, triglycerides, total and direct bilirubin, aspartate aminotransferase, alanine aminotransferase (ALT), creatinine, blood urea nitrogen, total proteins and albumin were measured with a fully automated cobas® 6000 analyzer Roche (Roche Diagnostics, Mannheim, Germany).

Urine samples were collected in individual metabolic cages for a 12 h period at the end of the experimental period. This method allowed urine to be obtained without fecal contamination. The samples were used to record urine volume and other parameters, including specific gravity, pH, glucose, protein, ketones, bilirubin, urobilinogen and blood pigments, which were measured using test strips Bayer (Bayer Diagnostics, Buenos Aires, Argentina). The urine sediment was examined under a light microscope Nikon fluophot (Nippon Kogaku K.K., Tokyo, Japan) after urine centrifugation at 2236 × g for 10 min.

On the day following the last administration, the animals were fasted overnight but allowed access to water *ad libitum* and then killed under anesthesia (i.p. ketamine/xylazine (50: 5 mg kg^{-1}). All the organs were carefully examined macroscopically *in situ* and selected organs (liver, kidney, entire gastrointestinal tract, brain, lungs, heart, spleen, testes, prostate gland, uterus and ovaries) were excised, weighed and fixed in 10% neutral formaldehyde for histopathological examinations. 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 Laboratories, Inc., California, USA) stained with hematoxylin–eosin, examined at various magnifications to detected pathological lesions and photographed using a light photomicroscope (Nikon; digital camera, 2 MPx).

Subchronic oral toxicity study of pure crystalline polymatin A. A similar experimental design to that of the previous section was used to determine the subchronic toxicity of polymatin A. The rats were housed in cages and divided into four groups of 10 animals each (five of each sex): group I received polymatin A 7 mg kg^{-1} day^{-1}; group II received polymatin A 14 mg kg^{-1} day^{-1}; group III received polymatin A 28 mg kg^{-1} day^{-1}; and group IV received distilled water and was used as the control.

The appropriate amount of polymatin A 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 the 10% decoction (see *In vivo Toxicity studies- Subchronic oral toxicity study of S. macroscyphus leaves*).

Statistical analysis

The data of the *in vitro* effects were analyzed by one-way analysis of variance using SPSS software version 12.0. A 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, statistical significance was evaluated by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Phytochemical analysis of the aqueous extract of *S. macroscyphus* leaves

The IR spectroscopy of the 10% decoction showed strong absorptions at 3300 cm^{-1} (phenol O-H stretch) and at 1600 cm^{-1} (alkenyl C=C stretch), indicating the presence of phenolic compounds in the extract. In addition, absorption corresponding to γ-lactone carbonyl (1755–1780 cm^{-1}) was observed in the IR spectrum, indicating that SLTs are also present in the extract.

HPLC chromatography of the extract, showed peaks corresponding to 3-caffeoylquinic acid, caffeic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5 dicaffeoylquinic acid. HPLC and GC-MS analysis allowed identification of the lactone polymatin A as a component in the decoction.

Chemical characterization of polymatin A isolated from *S. macroscyphus* leaves

Polymatin A was obtained by leaf soaking in chloroform as described in Materials and methods. This procedure was effective to purify preparative amounts of polymatin A, yielding 0.71% (w/w of dry leaf) of crystalline lactone. GC-MS analysis indicated a purity of 97.4% of polymatin A. H NMR (300 MHz, $CDCl_3$) δ in ppm (J in Hz; assignment): 6.85 dd (9.9 and 7.3 Hz; H-1), 6.33 dd (8.4 and 1.8; H-8), 6.24 d (3.2; H-13a); 6.11 qq (7.1 and 1.4; H-3′); 5.67 d (3.4; H-13b); 5.08 dd (10.5 and 9.5; H-6); 4.94 broad d (10.5; H-5); 4.00 dd (10 and 8.4; H-9), 3.80 s (3H; $-CO_2Me$); 2.82 d (10; –OH), 2.65 dddd (9.5, 3.4, 3.2 and 1.8; H-7), 2.48 m (H-2a), 2.33 m (H-3b), 2.24 m (H-2b), 2.09 m (H-3a), 1.98 dq (3H; 7.3 and 1.4; H-4′), 1.88 broad s (6H; H-15 and H-5′).

^{13}C NMR data follow: ^{13}C NMR (75 MHz, $CDCl_3$): 169.21 (C-12), 167.42 (C-14), 166.84 (C-1′), 144.19 (C-1), 139.46 (C-3′), 137.58 (C-4), 134.77 (C-11), 133.71 (C-10), 126.89 (C-2′), 126.40 (C-5), 121.09 (C-13), 75.56 (C-6), 71.65 (C-8), 71.08 (C-9), 52.11 (OMe), 51.42 (C-7), 36.65 (C-3), 25.71 (C-2), 20.40 (C-5′), 16.81 (C-15), 15.75 (C-4′). MS (EI, 70 eV, GC-MS): m/z (%) M^+ 390 (0.06), 359 (0.1), 307 (1), 290 (1), 272 (1), 258 (3), 240 (2), 212 (3), 193 (2), 128 (3), 105 (4), 83 (100), 44 (43), 0.06] (38), 211 (43), 167 (8), 151 (11), 149 (4), 125 (28), 109 (100), 108 (9), 83 (26), 82 (15), 81 (13), 79 (11), 67. HRMS: M^+ 390.1684 (calcd for $C_{21}H_{26}O_7$ 390.1678). The ^1H NMR and ^{13}C NMR spectra matched exactly with the data reported previously (Serra-Barcellona *et al.*, 2014). Chemical structure is shown in Fig. 1.

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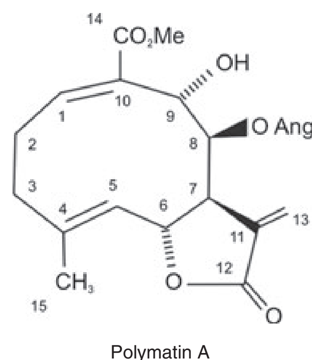

Figure 1. Chemical structure of polymatin A.

In vivo antidiabetic effect

Figure 2 shows the fasting blood glucose levels of the different experimental groups at the beginning and end of the experimental period. Diabetic animals treated with 140 mg kg^{-1} day^{-1} of 10% decoction of *S. macroscyphus* leaves (STZ-Sm) or 14 mg kg^{-1} day^{-1} of pure polymatin A (STZ-PA) significantly decrease plasma glucose levels after 30 days, compared with their own baseline pretreatment and compared to the diabetic untreated group (STZ) ($P < 0.05$). The standard drug, glimepiride (5 mg kg^{-1} day^{-1}) also significantly reduced ($P < 0.05$) blood glucose levels of STZ diabetic rats during this period.

In vitro cytotoxicity study

The effect of different concentrations of 10% decoction of *S. macroscyphus* (0.4, 4, 40, 100 and 200 μg ml^{-1} of dry extract) and pure polymatin A (0.12, 1.2, 12, 30 and 60 μg ml^{-1} of polymatin A) on Hep-G2, COS1, CHO-K1 and Vero cells culture was studied.

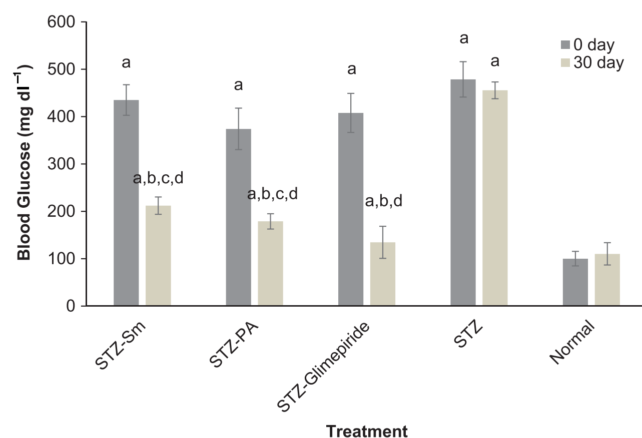

Figure 2. Effect of treatment with 10% decoction of *S. macroscyphus* leaves and polymatin A on fasting glucose levels in blood of diabetic rats at the beginning and end of the experimental period (4 weeks). The histogram values indicate the mean ± SD of the blood glucose of the different groups studied. $^{a}P < 0.05$ compared with the normal rats (Normal). $^{b}P < 0.05$ compared with diabetic rats (STZ). $^{c}P < 0.05$ compared to STZ diabetic treated with glimepiride (STZ-Glimepiride). $^{d}P < 0.05$ compared to pretreatment levels within the same group. STZ, STZ diabetic group; STZ-Sm, STZ diabetic treated with 10% decoction of *S. macroscyphus* leaves (140 mg kg^{-1} body weight); STZ-PA, STZ diabetic treated with polymatin A (14 mg kg^{-1} body weight); STZ-Glimepiride, STZ diabetic treated with glimepiride (5 mg kg^{-1} body weight). STZ, streptozotocin.

The MTT assay evidenced that the aqueous and polymatin A extracts showed cytotoxicity, with different IC_{50} values, when compared to the control ($P < 0.001$). The concentrations at which cell viability was inhibited to 50% of control values (IC_{50}) were 22, 30, 128 and 109 μg ml^{-1} of 10% decoction for Hep-G2, CHO-K1, COS1 and Vero cells, respectively. IC_{50} values for polymatin A were 1.94, 2.6, 12.2 and 11.8 μg ml^{-1} for Hep-G2, CHO-K1, COS1 and Vero cells, respectively.

Subchronic toxicity studies

Effect of subchronic oral administration of 10% decoction of S. macroscyphus leaves. In general terms, the oral daily administration of 10% decoction of *S. macroscyphus* leaves for 90 days, caused no signs of toxicity or deaths in rats at any tested dose (70, 140 and 280 mg kg^{-1} day^{-1}). No significant clinically relevant changes were observed in general behavior or physiological activities.

During the course of the experiment, no significant differences between the control and any of the treated groups were recorded in absolute body weight. All groups of rats treated with the 10% decoction gained weight with time in similar ways (35.65 ± 7.61 g for group I; 33.10 ± 8.02 g for group II; 33.58 ± 6.63 g for group III; and 47.11 ± 8.23 g for group IV; $P > 0.05$). In addition, no differences in daily food and water intake during the experimental period were observed (data not shown).

In the present study, no significant changes in hematological parameters were noticed at 30 and 60 days (data not shown) or at the end of the experimental period in male- or female-treated rats compared to the control groups (Table 1). The administration of 10% decoction did not induce statistical changes in fasting glucose, total cholesterol, triglycerides, urea, creatinine, total and direct bilirubin, total proteins or albumin serum levels throughout the treatment. None of the doses tested caused alterations in the activity of the enzymatic markers of liver injury (aspartate aminotransferase and ALT) at any time during the experimental period. Biochemistry values after 90 days of treatment with *S. macroscyphus* leaves decoction are summarized in Table 2.

No significant differences between treated and untreated (control) rats were observed in the urine parameters from male and female rats (Table 3). In addition, microscopic examination of the urine sediment did not show any abnormal cells (leukocytes, erythrocytes or renal epithelial cells), casts, crystals or microorganisms. As shown in Table 3, sediment was scarce. At the end of the experimental period, macroscopic observation *in situ* of the different organs (liver, kidney, entire gastrointestinal tract, brain, lungs, heart, spleen, testes, prostate gland, uterus and ovaries) showed no alterations due to treatment with different doses of 10% decoction. Table 4 shows the results for absolute and relative weights of selected organs (liver, kidney and gastrointestinal tract) after 90 days of treatment with 10% decoction. Statistical analyses of the data indicate that there were no treatment-related variations in either absolute organ weights or when these weights were expressed in relation to body weight. The histopathological examination of these organs did not reveal any microscopic lesions related to the treatment showing a histoarchitecture comparable to that of the untreated control group. Figure 3 shows representative photomicrographs of the liver, kidney and intestine from rats assayed at the maximum dose (280 mg kg^{-1} day^{-1} of dry extract of 10% decoction of *S. macroscyphus*). No signs of damage were observed in any of the animals after 90 days of treatment (Fig. 3A). Hepatocytes are relatively uniform in size and shape, without lymphocytic aggregates or sinusoidal congestion. The kidneys of

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Table 1. Effect of subchronic oral administration of 10% decoction of *Smallanthus macroscyphus* leaves on hematological parameters. Terminal blood values after 90 days of treatment

Hematological parameters	Group I (70 mg kg^{-1} day^{-1})	Group II (140 mg kg^{-1} day^{-1})	Group III (280 mg kg^{-1} day^{-1})	Group IV (control)
Red blood count (10^6 μl^{-1})	7.40 ± 0.13	7.30 ± 0.59	7.23 ± 0.18	6.83 ± 0.99
Hemoglobin (g dl^{-1})	13.14 ± 0.39	12.77 ± 1.33	13.57 ± 0.81	12.51 ± 0.56
Hematocrit (%)	61.34 ± 1.95	60.53 ± 1.86	58.17 ± 1.10	59.07 ± 9.01
Mean corpuscular volume (fl)	84.33 ± 1.54	85.27 ± 0.95	82.09 ± 2.24	86.42 ± 2.21
Mean corpuscular hemoglobin (pg)	18.30 ± 0.80	19.23 ± 0.32	18.78 ± 0.73	19.60 ± 0.55
Mean corpuscular hemoglobin concentration (g dl^{-1})	22.98 ± 0.51	22.53 ± 0.15	23.34 ± 0.30	22.73 ± 0.56
White blood count (10^3 μl^{-1})	7.45 ± 2.11	6.22 ± 1.46	6.35 ± 0.66	7.86 ± 4.84
Neutrophils (10^3 μl^{-1})	1.62 ± 0.29	1.40 ± 0.50	1.56 ± 0.37	1.54 ± 0.37
Lymphocytes (10^3 μl^{-1})	5.06 ± 2.27	4.03 ± 1.50	4.23 ± 0.66	5.65 ± 4.58
Monocytes (10^3 μl^{-1})	0.24 ± 0.11	0.32 ± 0.23	0.11 ± 0.13	0.22 ± 0.17
Eosinophils (10^3 μl^{-1})	0.31 ± 0.13	0.27 ± 0.17	0.28 ± 0.19	0.15 ± 0.08
Basophils (10^3 μl^{-1})	0.22 ± 0.06	0.20 ± 0.12	0.17± 0.08	0.30 ± 0.23
Platelet count (10^3 μl^{-1})	968.05 ± 63.57	865.75 ± 84.3	890.12 ± 86.26	985.67 ± 11.61

Data are mean ± SD. $n = 10$ animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV).

Table 2. Effect of subchronic oral administration of 10% decoction of *Smallanthus macroscyphus* leaves on blood biochemical parameters. Terminal biochemistry values after 90 days of treatment

Blood biochemical parameters	Group I (70 mg kg^{-1} day^{-1})	Group II (140 mg kg^{-1} day^{-1})	Group III (280 mg kg^{-1} day^{-1})	Group IV (control)
Glucose (mg dl^{-1})	94.00 ± 2.37	88.00 ± 1.73	102.5 ± 4.12	93.55 ± 5.25
Total cholesterol (mg dl^{-1})	78 ± 4.87	97 ± 7.65	83 ± 8.49	82.13 ± 9.29
Triglycerides (mg dl^{-1})	54.42 ± 4.03	69.00 ± 8.49	49.00 ± 5.66	65.51 ± 19.09
Urea (g l^{-1})	0.38 ± 0.06	0.40 ± 0.09	0.35 ± 0.07	0.43 ± 0.04
Creatinine (mg dl^{-1})	0.23 ± 0.04	0.31 ± 0.05	0.27 ± 0.04	0.24 ± 0.02
Total proteins (g dl^{-1})	6.93 ± 0.25	6.77 ± 0.42	6.64 ± 0.15	6.61 ± 0.30
Albumin (g dl^{-1})	4.13 ± 0.05	4.00 ± 0.11	3.97 ± 0.17	4.22 ± 0.12
AST (UI l^{-1})	177.14 ± 16.83	192.52 ± 17.78	146.51 ± 18.36	206.03 ± 39.43
ALT (UI l^{-1})	58.56 ± 1.29	52.33 ± 4.62	49.98 ± 4.88	57.33 ± 2.58

ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Data are mean ± SD. $n = 10$ animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV).

treated animals show normal glomeruli without vascular congestion or leukocyte infiltrations and regular tubular cells with normal nuclei (Fig. 3B), whereas section of intestine shows normal epithelial cells leaning on the lamina propia in each intestinal villus (Fig. 3C).

Effect of the subchronic oral administration of pure polymatin A

No toxicity signs or deaths were recorded during the 90 consecutive days of oral treatment with polymatin A at any tested dose. Body weight gain was not affected by the administration of pure lactone at any of the doses assayed (33.88 ± 6.87 g for group I; 33.35 ± 8.65 g for group II; 34.10 ± 5.43 g for group III; and 47.11 ± 8.23 g for group IV; $P > 0.05$).

Moreover, treatment with different doses of polymatin A produced no significant changes in daily food intake (data not shown) or in water consumption. The hematological and blood biochemical parameters measured after the 90-day administration of different doses of polymatin A remained within physiological ranges, without significant differences with the control group (Tables 5 and 6). Additionally, no significant changes were noted on days 30 and 60 of treatment (data not shown). The urinary parameters determined remained within physiological values after a treatment period of 90 days, as shown in Table 7.

Macroscopic examination *in situ* of vital organs (liver, kidney, entire gastrointestinal tract, brain, lungs, heart, spleen, testes, prostate gland, uterus and ovaries) revealed no abnormalities compared with control animals. Similarly to treatment with decoction, the oral administration of the lactone at different doses assayed for a period of 90 days did not produce treatment-related alterations in either the absolute or the relative weight of selected organs (Table 8). The histological evaluation did not reveal any lesions or pathological changes in these organs that could be

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Table 3. Effect of subchronic oral administration of 10% decoction of *Smallanthus macroscyphus* leaves on urine parameters. Terminal values after 90 days of treatment

Urine parameters	Group I (70 mg kg^{-1} day^{-1})	Group II (140 mg kg^{-1} day^{-1})	Group III (280 mg kg^{-1} day^{-1})	Group IV (control)
Volume (ml per 24 h)	9.76 ± 2.13	10.13 ± 2.42	7.23 ± 2.25	10.5 ± 3.07
Specific gravity	1012.5 ± 5.0	1012.5 ± 5.0	1013.7 ± 7.5	1012.5 ± 6.8
pH	7.61 ± 0.52	8.12 ± 0.25	8.25 ± 0.87	8.25 ± 0.53
Glucose (mg dl^{-1})	ND	ND	ND	ND
Proteins (mg dl^{-1})	$<$ 20.0	$<$ 20.0	$<$ 20.0	$<$ 20.0
Ketones (mg dl^{-1})	ND	ND	ND	ND
Bilirubin (mg dl^{-1})	ND	ND	ND	ND
Urobilinogen (mg dl^{-1})	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
Blood pigments (mg dl^{-1} hemoglobin)	ND	ND	ND	ND
Sediment	Scarce	Scarce	Scarce	Scarce

ND, not detected.
Data are mean ± SD. n = 10 animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV).

Table 4. Effect of subchronic oral administration of 10% decoction of *Smallanthus macroscyphus* leaves on terminal body weight and absolute-relative organ weights

	Group I (70 mg kg^{-1} day^{-1})	Group II (140 mg kg^{-1} day^{-1})	Group III (280 mg kg^{-1} day^{-1})	Group IV (control)
Body weight (g)	239.00 ± 20.76	242.25 ± 37.96	246.25 ± 21.75	265.67 ± 73.65
Body weight gain (g)	35.65 ± 7.61	33.10 ± 8.02	33.58 ± 6.63	47.11 ± 8.23
Liver weight (g)	7.65 ± 1.78	7.98 ± 1.62	8.11 ± 1.53	8.66 ± 0.17
LW/BW ratio (%)	3.20 ± 0.32	3.29 ± 0.17	3.29 ± 0.40	3.25 ± 0.88
Kidney weight (g)	1.89 ± 0.26	1.94 ± 0.32	1.96 ± 0.18	1.86 ± 0.24
KW/BW ratio (%)	0.79 ± 0.03	0.80 ± 0.05	0.79 ± 0.02	0.70 ± 0.10
Gastrointestinal weight (g)	9.93 ± 0.92	10.44 ± 0.74	10.6 ± 1.62	11.19 ± 1.86
GIW/BW ratio (%)	4.15 ± 0.41	4.30 ± 0.57	4.30 ± 0.66	4.21 ± 0.71

Data are mean ± SD. n = 10 animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV). LW, liver weight; BW, body weight; KW, kidney weight; GIW, gastrointestinal weight.

attributable to treatment with STL, even at the highest dose tested. Figure 3(C,F,I) shows representative photomicrographs of the liver, kidney and intestine in rats after 90 days of oral administration of polymatin A at the maximum dose assayed. No treatment-related tissue injuries were observed in these organs such as cell degeneration, necrosis or inflammatory reactions.

Discussion

The toxicity/safety profile of both 10% decoction of *S. macroscyphus* leaves and their major active lactone, polymatin A, was determined to implement new active compounds from natural sources for the treatment of diabetes mellitus.

Interestingly, in a previous work we demonstrated that *S. macroscyphus* leaves have chemical compounds exhibiting significant hypoglycemic and antidiabetic properties (Serra-Barcellona *et al.*, 2014). The current study demonstrated that the 10% decoction of this plant and isolated polymatin A have a safety margin in rats at doses in which hypoglycemic effects were determined.

It is well known that plant extracts containing a very complex mix of chemical compounds may cause adverse effects (Jordan *et al.*, 2010). HPLC analysis of the 10% decoction of *S. macroscyphus* leaves using different solvent programs has revealed the presence of caffeic acid, 3-caffeoylquinic acid, three dicaffeoil quinic acids (3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5 dicaffeoylquinic acid) and the lactone polymatin A (Serra-Barcellona *et al.*, 2014). Although the phenolic compounds have not shown major toxic effects (Chow *et al.*, 2003), a few studies have demonstrated that STLs may cause acute or chronic toxicity, which is the basis for their potential anticancer use (Cotugno *et al.*, 2012; De Ford *et al.*, 2015; Schmidt, 1999; Zhang *et al.*, 2005). In most cases, STLs have poor toxicology profiles and little is known about their general unwanted toxicity toward normal cells (Ghantous *et al.*, 2010). Consequently, it was a matter of interest to evaluate the potential toxic effects of a wide range of doses of polymatin A in healthy adult rats.

In the present paper, we conducted an *in vitro* test to evaluate potential toxicities using four different cell lines. Cell line selection was based on the main target organs of the potential adverse or

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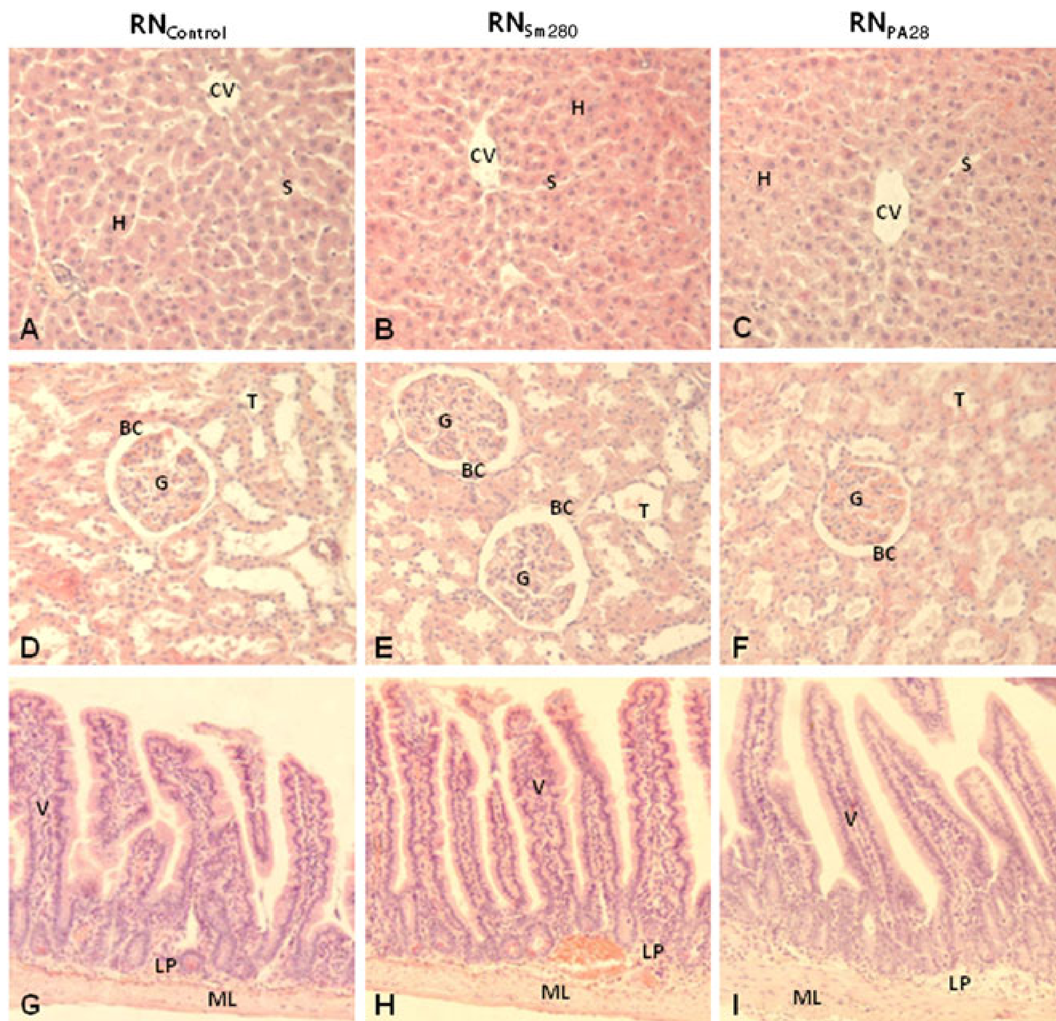

Figure 3. Representative photomicrographs from histological sections of liver (A–C), kidney (D–F) and intestine (G–I) of control rats (A, D and G), orally treated rats with a 10% decoction of S. macroscyphus leaves at a dose of 280 mg kg^{-1} day^{-1} (B, E and H) and pure polymatin A at a dose of 28 mg kg^{-1} day^{-1} (C, F and I) for a period of 90 days. Histological sections of each organ were stained with hematoxylin–eosin. A–F, × 400; G–I, × 126. BC, Bowman's capsule; CV, central vein; G, glomerulus; H, hepatocytes; LP, lamina propria; ML, muscle layer; S, sinusoid; T, tubule; V, villi.

toxic effects of the extract or pure compound under investigation. CHO-K1 cells, which are derived from the ovary of adult hamsters, have the morphology of epithelial cells. Hep-G2 cells are used in studies of the liver toxicity of xenobiotics because it has a liver-like enzyme pattern. COS 1 is a fibroblast-like cell line from monkey kidney tissue and Vero is a normal epithelial cell line from the kidney of adult monkeys. The MTT assay showed a concentration-dependent decrease in mitochondrial function and cell viability because of the treatment. COS 1 and Vero cells being the most resistant ones to treatment with the 10% decoction, showing similar IC_{50}, whereas CHO-K1 and Hep-G2 showed a lower IC_{50}. A similar behavior was observed with cells treated with polymatin A, COS 1 and Vero cells being the most resistant ones to this treatment. It should be noted that cell lines *in vitro* could respond to the same toxic insult differently from cells in the intact organ *in vivo*. It is known that the *in vivo* effects of an oral dose are subject to systemic bioavailability and hepatic metabolism, these pharmacokinetic processes being absent in a cell culture model (Singh, 2006).

In a previous work we determined the absence of acute toxic *in vivo* effects of the 10% decoction of *S. macroscyphus* leaves and of polymatin A at all doses up to the highest dose tested, suggesting that the lethal dose 50 would be above 14.0 and 2.8 g kg^{-1} body weight, respectively. These doses are significantly higher than the effective hypoglycemic doses (Serra-Barcellona *et al*., 2014). We think that the difference between the doses in the present *in vitro* and in previous *in vivo* studies may be attributed to the different effective cellular concentrations of the active compounds. In a recent review, Schrage *et al.* (2011) concluded that the use of cytotoxicity data alone did not sufficiently contribute to the study of the safety of compounds.

Therefore, our results of *in vitro* studies using several cell lines were considered as an initial survey to evaluate potentially toxic doses of the extract or pure compound of *S. macroscyphus* leaves and represent a complement of the *in vivo* studies.

Subchronic toxicity studies are important tools in evaluating the safety profile of herbal medicines or chemical compounds isolated from plants, given that acute toxicity studies are not sufficient to describe a safety profile effectively. In the present work, we adopted a 90-day toxicity study to assess the safety profile of the 10% decoction of *S. macroscyphus* and polymatin A, which is a well-accepted method for evaluating toxicity in long-term drug administration (Lahlou *et al*., 2008; Serra-Barcellona *et al*., 2012).

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Table 5. Effect of subchronic oral administration of polymatin A on hematological parameters. Terminal blood values after 90 days of treatment

Hematological parameters	Group I (7 mg kg^{-1} day^{-1})	Group II (14 mg kg^{-1} day^{-1})	Group III (28 mg kg^{-1} day^{-1})	Group IV (control)
Red blood count (10^6 μl^{-1})	7.14 ± 0.28	7.54 ± 0.21	7.31 ± 0.31	6.83 ± 0.99
Hemoglobin (g dl^{-1})	12.67 ± 0.76	13.20 ± 0.16	13.11 ± 0.34	12.51 ± 0.56
Hematocrit (%)	59.86 ± 3.21	62.05 ± 0.78	60.57 ± 2.05	59.07 ± 9.01
Mean corpuscular Volume (fl)	82.77 ± 1.69	83.33 ± 2.48	83.12 ± 3.49	86.42 ± 2.21
Mean corpuscular hemoglobin (pg)	19.03 ± 0.43	18.57 ± 0.57	18.93 ± 0.67	19.60 ± 0.55
Mean corpuscular hemoglobin concentration (g dl^{-1})	22.46 ± 0.35	22.70 ± 0.28	22.72 ± 0.52	22.73 ± 0.56
White blood count (10^3 μl^{-1})	7.74 ± 1.65	8.33 ± 2.87	6.41 ± 1.02	7.86 ± 4.84
Neutrophils (10^3 μl^{-1})	1.59 ± 0.42	1.77 ± 0.43	1.62 ± 0.25	1.54 ± 0.37
Lymphocytes (10^3 μl^{-1})	5.60 ± 1.63	5.96 ± 1.29	4.24 ± 0.78	5.65 ± 4.58
Monocytes (10^3 μl^{-1})	0.18 ± 0.13	0.12 ± 0.10	0.14 ± 0.02	0.22 ± 0.17
Eosinophils (10^3 μl^{-1})	0.22 ± 0.05	0.38 ± 0.21	0.33 ± 0.20	0.15 ± 0.08
Basophils (10^3 μl^{-1})	0.15 ± 0.03	0.10 ± 0.09	0.10 ± 0.05	0.30 ± 0.23
Platelet count (10^3 μl^{-1})	934.12 ± 52.38	951.54 ± 48.49	957.51 ± 56.26	985.67 ± 11.61

Data are mean ± SD. $n = 10$ animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV).

Table 6. Effect of subchronic oral administration of polymatin A on blood biochemical parameters. Terminal biochemistry values after 90 days of treatment

Blood biochemical parameters	Group I (7 mg kg^{-1} day^{-1})	Group II (14 mg kg^{-1} day^{-1})	Group III (28 mg kg^{-1} day^{-1})	Group IV (control)
Glucose (mg dl^{-1})	91.34 ± 3.02	92.25 ± 4.24	89.59 ± 2.12	93.55 ± 5.25
Total cholesterol (mg dl^{-1})	83.10 ± 3.87	80.97 ± 4.04	71.99 ± 2.65	82.13 ± 9.29
Triglycerides (mg dl^{-1})	46.52 ± 12.13	66.23 ± 13.00	55.33 ± 11.51	65.51 ± 19.09
Urea (g l^{-1})	0.40 ± 0.08	0.40 ± 0.10	0.36 ± 0.06	0.43 ± 0.04
Creatinine (mg dl^{-1})	0.26 ± 0.06	0.24 ± 0.04	0.27 ± 0.03	0.24 ± 0.02
Total proteins (g dl^{-1})	7.03 ± 0.15	7.10 ± 0.28	7.54 ± 0.71	6.61 ± 0.30
Albumin (g dl^{-1})	4.08 ± 0.06	4.51 ± 0.18	4.65 ± 0.35	4.22 ± 0.12
AST (UI l^{-1})	165.34 ± 4.73	174.23 ± 5.66	159.5 ± 8.94	206.03 ± 39.43
ALT (UI l^{-1})	53.66 ± 3.28	60.67 ± 6.51	52.32 ± 2.83	57.33 ± 2.58

ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Data are mean ± SD. $n = 10$ animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV).

The study design in this paper used the clinically relevant oral route and a daily administration, as was employed in a previous study to verify the preclinical antidiabetic effect (Serra-Barcellona *et al.*, 2014). In addition, the administration of test products before feeding with an intragastric tube is more accurate than dietary administration and probably represents a higher absorbed dose (Singh, 2006).

The results show that the aqueous extract of *S. macroscyphus* leaves orally administered is non-toxic, at least up to the maximum level assayed (280 mg kg^{-1} day^{-1}). Similarly, polymatin A, the main STL isolated from the leaves, has no toxic effects even at the highest dose tested (28 mg kg^{-1} day^{-1}). All groups of treated animals survived the 90-day treatment period and the absence of toxic effects was demonstrated by clinical signs and behavior evaluations of the animals, blood and urine biochemical analyses and macroscopic/histopathological examinations of selected organs. After exposure to toxic compounds, there is usually a reduction in body weight gain as well as in food and water consumption (Rosidah *et al.*, 2009; Teo *et al.*, 2002). In our subchronic experiment, all groups of rats treated with decoction or polymatin A showed no significant changes in daily food intake or in water consumption. In addition, the treated animals gained weight with time and no significant alterations were recorded in absolute or relative organ weight, demonstrating the lack of negative effects on these parameters, at least within the range of the doses assayed.

Hematopoietic organs are readily affected by a wide variety of substances. It is, therefore, understandable that hematological tests should be one of the routine procedures performed in the assessment of safety profile of various chemical compounds (Fliedner *et al.*, 1990). In the present study, none of the *in vivo* treatments with either 10% decoction or polymatin A at any of the doses tested induced quantitative alterations in circulating red blood cell, leukocytes or platelets. These findings could indicate the absence of hematopoietic toxicity.

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Table 7. Effect of subchronic oral administration of polymatin A on urine parameters. Terminal values after 90 days of treatment

Urine parameters	Group I (7 mg kg^{-1} day^{-1})	Group II (14 mg kg^{-1} day^{-1})	Group III (28 mg kg^{-1} day^{-1})	Group IV (control)
Volume (ml per 24 h)	8.24 ± 1.67	6.35 ± 2.71	7.81 ± 2.97	10.5 ± 3.07
Specific gravity	1012.5 ± 5.2	1021.5 ± 6.3	1019.0 ± 6.29	1012.5 ± 6.8
pH	8.25 ± 0.87	7.75 ± 0.64	7.62 ± 0.48	8.25 ± 0.53
Glucose (mg dl^{-1})	ND	ND	ND	ND
Proteins (mg dl^{-1})	< 20.0	< 20.0	< 20.0	< 20.0
Ketones (mg dl^{-1})	ND	ND	ND	ND
Bilirubin (mg dl^{-1})	ND	ND	ND	ND
Urobilinogen (mg dl^{-1})	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
Blood pigments (mg dl^{-1} hemoglobin)	ND	ND	ND	ND
Sediment	Scarce	Scarce	Scarce	Scarce

ND, not detected.
Data are mean ± SD. $n = 10$ animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV).

Table 8. Effect of subchronic oral administration of polymatin A on terminal body weight and absolute-relative organ weights

	Group I (7 mg kg^{-1} day^{-1})	Group II (14 mg kg^{-1} day^{-1})	Group III (28 mg kg^{-1} day^{-1})	Group IV (control)
Body weight (g)	236.00 ± 4.06	239.00 ± 3.12	234.25 ± 8.55	265.67 ± 73.65
Body weight gain (g)	33.88 ± 6.87	33.35 ± 8.65	34.10 ± 5.43	47.11 ± 8.23
Liver weight (g)	7.84 ± 1.16	7.79 ± 1.19	7.34 ± 1.22	8.66 ± 0.17
LW/BW ratio (%)	3.32 ± 0.24	3.12 ± 0.12	3.13 ± 0.37	3.25 ± 0.88
Kidney weight (g)	1.87 ± 0.31	1.97 ± 0.23	1.99 ± 0.17	1.86 ± 0.24
KW/BW ratio (%)	0.79 ± 0.09	0.82 ± 0.06	0.85 ± 0.07	0.70 ± 0.10
Gastrointestinal weight (g)	10.12 ± 0.96	9.68 ± 0.26	9.85 ± 1.36	11.19 ± 1.86
GIW/BW ratio (%)	4.29 ± 0.60	4.05 ± 0.50	4.20 ± 0.59	4.21 ± 0.71

Data are mean ± SD. $n = 10$ animals (five males and five females) per group. $P < 0.05$ was considered statistically significant compared with control group (group IV). LW, liver weight; BW, body weight; KW, kidney weight; GIW, gastrointestinal weight.

It has been shown that severe hypoglycemia is strongly associated with increased risks of adverse clinical outcomes in diabetic patients receiving an intensive glucose-lowering intervention (Zoungas *et al.*, 2010). The results presented here indicated a lack of adverse hypoglycemic effect in normal rats at any of the doses tested of decoction or polymatin A.

Chemical-mediated hepatic injury is generally indicated by elevations in serum aminotransferase levels, but may not always reveal clinically significant liver damage. This is because of the great capacity of the liver to heal injury, whereas increases in both total and conjugated bilirubin levels are measures of overall excretory liver function (Navarro & Senior, 2006). Treatment with a decoction of *S. macroscyphus* leaf or polymatin A did not cause any changes in serum aminotransferase levels, mainly in ALT, the most reliable indicator to detect both acute and subacute hepatocellular injury (Meyer & Harvey, 2004). In addition, the levels of direct and total bilirubin showed normal values, reflecting the liver's ability to move bilirubin from plasma into bile, and therefore a conserved excretory liver function. Another measurable liver function is protein synthesis. In the present work, the acceptable function of the liver was also reflected in the normal serum albumin concentration detected in treated rats. Even more, the absence of vague symptoms in the treated animals such as inactivity, anorexia, emesis or dark urine, in conjunction with the lack of histopathological lesions or changes in either absolute or relative liver weight, strongly indicated that the assayed treatments did not affect this important organ.

The kidney is another organ vulnerable to injury from a variety of substances with toxic potential (Perazella, 2009). Early manifestations of renal injury are tubular necrosis, electrolyte imbalances and mild urinary sediment until an evident change in renal functional capacity is measured as an increase in blood urea and creatinine (Pannu & Nadim, 2008).

In the experimental conditions of this study, no renal function parameter was affected by treatments with 10% decoction or polymatin A. Additionally, the treated animals showed no renal edema, neither tubular or glomerular lesions nor inflammatory cell infiltration, suggesting that orally administered products, at least up to the maximum level assayed, did not affect the kidney either histologically or functionally.

This study was undertaken to detect potential damage in the gastrointestinal tract after the oral administration of *S. macroscyphus* decoction or polymatin A in rats as a way to enlarge their toxicological profiles. No histological alterations along the gastrointestinal tract associated with the long-term consumption of these products were observed and no stomach or intestinal

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hypertrophy was found. These are highly interesting results that strongly support the safe use of the 10% decoction and of polymatin A because it is known that oral ingestion of several chemical compounds may result in serious gastrointestinal side effects in both the upper and lower tract because of local and/or systemic action (Yáñez *et al.*, 2003).

Conclusions

The efficacy and safety of extracts or active compounds of *S. macroscyphus* were studied thoroughly to maximize their therapeutic benefits. Previously we established in rats the effective hypoglycemic dose of the 10% decoction of *S. macroscyphus* leaves and polymatin A, the main STL isolated from them (Serra-Barcellona *et al.*, 2014). Based on the toxicological parameters evaluated in the current study, we conclude that decoction and polymatin A are safe when it is orally administered to rats at doses that the antidiabetic effect has been demonstrated.

Conflict of interest

The authors did not report any conflict of interest.

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