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Research Paper

In-vivo laxative and toxicological evaluation and in-vitro antitumour effects of *Mitracarpus frigidus* aerial parts

Rodrigo Luiz Fabri[a], Danielle Maria de Oliveira Aragão[a], Jônatas Rodrigues Florêncio[a], Gabriele Mendes Matos Cardoso[c], Elaine Maria de Souza-Fagundes[c], Maria Christina Marques Nogueira Castanon[b] and Elita Scio[a]

[a]Bioactive Natural Products Laboratory, Department of Biochemistry, and [b]Department of Histology, Biological Sciences Institute, Federal University of Juiz de Fora, Juiz de Fora and [c]Department of Physiology and Biophysics, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Brazil

Keywords
antitumour; intestinal motility; laxative; *Mitracarpus frigidus*; toxicity

Correspondence
Elita Scio, Bioactive Natural Products Laboratory, Department of Biochemistry, Biological Sciences Institute, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, CEP 36036 900, Brazil.
E-mail: elita.scio@ufjf.edu.br

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Abstract

Objectives To evaluate the in-vitro antitumour properties, and the in-vivo laxative and toxicological effects of the methanolic extract of the aerial parts of *Mitracarpus frigidus* (MFM).

Methods The in-vitro antitumour activity of MFM was evaluated against three human tumour cell lines: Jurkat, HL60 and MCF-7. The laxative activity and the effect of MFM on intestinal motility were evaluated in rats at the doses of 100, 300 and 1000 mg/kg. Acute oral toxicity was performed at 10, 100, 1000 and 2000 mg/kg and subchronic toxicity was evaluated at 100, 300 and 1000 mg/kg of MFM during a 42-day period. After subchronic administration of MFM the biochemical, haematological and histopathological parameters were analysed. Also, the total content of anthraquinones was determined.

Key findings MFM was cytotoxic only against HL60 and Jurkat cells with 89 and 83% growth inhibition, respectively. The laxative activity of MFM was similar to bisacodyl. Regarding the effect on intestinal motility, MFM showed a significant increase in the pathway of charcoal compared with the group treated with saline. Furthermore, MFM showed no in-vivo toxicity at the doses tested. Free and anthraquinone *C*- and *O*-glycosides were detected in MFM.

Conclusions MFM showed significant antitumour activity for leukaemic cells. Moreover, it presented laxative potential and no in-vivo toxicity.

Introduction

The medicinal plants used to treat many diseases are associated with folk medicine from different parts of the world.[1] Different cultures know and use the therapeutic potential of plants in the treatment of disease, and it is a practice that has evolved over centuries.[2]

Some species of the genus *Mitracarpus* (Rubiaceae) have many ethnopharmacological uses. For example, *Mitracarpus scaber* Zucc. ex Schult. & Schult. F. is used in traditional medicine in West Africa for headaches, toothache, amenorrhoea, dyspepsia, hepatic and venereal diseases, and leprosy. The juice of the plant is applied topically for the traditional treatment of diseases of the skin.[3] Germano *et al.*[4] reported the hepatoprotective effects of a decoction of *M. scaber* on CCl_4-induced hepatotoxicity *in vivo*, as well as *in vitro*, using isolated hepatocytes.

There are no reports however on the traditional uses of *M. frigidus* (Willd. ex Roem. & Schult.) K. Shum, an annual shrub commonly found in Brazil. However, the methanolic extract of the aerial parts of *M. frigidus* has shown significant leishmanicidal activity against *Leishmania chagasi* and *L. amazonensis* promastigote forms. Also, it has shown significant growth inhibition of pathogenic bacteria and yeasts strains.[5] Phytochemical screening of this extract revealed the presence of alkaloids, terpenoids, steroids, saponins, anthraquinones and phenolic compounds.[6]

This study has evaluated the in-vivo laxative and toxicological effects, and the in-vitro antitumour properties of the methanolic extract of the aerial parts of *M. frigidus*. The total content of anthraquinones was determined also.

Materials and Methods

Plant material

M. frigidus aerial parts were collected in Juiz de Fora, Minas Gerais, Brazil, in May, 2009. The plant was identified by Dr Tatiana Konno. A voucher specimen (CESJ 46076) was deposited at the Herbarium Leopoldo Krieger of Federal University of Juiz de Fora.

Preparation of the extract

The aerial parts (1 kg) of *M. frigidus* were powdered and macerated with methanol (5 × 2000 ml) for five days at room temperature. After evaporation of the solvent under reduced pressure at 45°C, the methanolic extract was obtained (MFM), and kept in tightly stoppered bottles under refrigeration until used for the biological testing and phytochemical assays.

Detection of free and combined anthraquinones

A portion of the MFM that was subjected to biological screening was used for the identification of free and conjugated anthraquinones, employing the protocols described by Matos.[7]

Quantitative analysis of total anthraquinones and total anthraquinone glycosides

The amounts of total anthraquinones and anthraquinone glycosides were determined as previously described by Sakulpanich and Gritsanapan[8] with slight modifications. The calibration curve was made using emodin as reference at five concentrations, from 1.56 to 25.00 μg/ml. The contents of total anthraquinones and total anthraquinone glycosides in MFM were calculated using the linear regression equation of emodin. The contents were expressed as mean ± standard deviation (SD) (% w/w).

In-vitro antitumour assay

Cell lines

Three human tumour cell lines were used, Jurkat (human immortalized line of T lymphocyte), HL60 (human promyelocytic leukaemia) and MCF-7 (breast cancer). HL60 and Jurkat cells were kindly furnished by Dr Gustavo Amarante-Mendes (São Paulo University, Brazil). MCF-7 was generously provided by Alfredo Goes (Federal University of Minas Gerais, Brazil). All lineages were maintained in the logarithmic phase of growth in RPMI 1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY, USA) enriched with 2 mM L-glutamine and 10% fetal bovine serum. All cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air. The media were changed twice weekly and they were regularly examined. All cell lines were used until 20 passages.

Evaluation of cytotoxic effect against human tumour cell lines

Tumour cell lines (Jurkat, HL60 and MCF-7) were inoculated at 50 000 cells per well. The plates were pre-incubated for 24 h at 37°C to allow adaptation of cells before addition of the samples. A freshly prepared solution of sample was tested for 48 h in an atmosphere of 5% CO_2 and 100% relative humidity. The samples included 20 μg/ml MFM, 0.05% dimethyl sulfoxide (DMSO; negative control) and 14 μM etoposide (positive control). Cell viability was estimated by measuring the rate of mitochondrial reduction of tetrazolium dye (MTT). The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only.[9] All samples were tested in triplicate, in three independent experiments.[10] The optical densities (OD) were measured with a spectrophotometer at 590 nm. Results were expressed as percentage of cell proliferation, comparing with 0.05% DMSO control and were calculated as follows: viability (%) = (mean OD treated – mean OD background)/mean OD untreated cultured, i.e. 0.05% DMSO – mean OD blank wells) × 100. Interactions of MFM and media were estimated on the basis of the variations between extract-containing medium and extract-free medium to avoid false-positive or false-negative results.[11]

DNA fragmentation assay

Cell cycle status and quantification of DNA fragmentation (hypodiploid DNA content) were performed by propidium iodide (PI) staining according to Nicolleti *et al*.[12] Cells were treated with MFM at 20 μg/ml in a 5% CO_2/95% air-humidified atmosphere at 37°C for 24 h. After incubation, the cells were centrifuged and re-suspended in hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated at 4°C for 4 h, and immediately analysed by flow cytometry. The PI fluorescence of 20 000 individual nuclei was measured using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data were analysed by FlowJo software (TreeStar Inc, CA, USA).

Animals

Male Wistar rats (60-days-old, 160–200 g) were obtained from the Reproduction Biology Center of the Federal University of Juiz de Fora. They were housed in a room kept under controlled conditions with temperature maintained at

22 ± 2°C, on a 12-h light/dark cycle, with free access to water and complete commercial chow (Nuvital, Colombo, PR, Brazil). Throughout the experiments animals were processed according to the suggested ethical guidelines for the care of laboratory animals. The study was approved by the Brazilian College of Animal Experimentation (COBEA – Protocol no. 009/2009).

Laxative activity

After acclimatization, rats previously fasted for 8 h were divided into five groups ($n = 6$) as follows: group A, negative control received normal saline + 3% DMSO; group B, reference group received bisacodyl (Dulcolax) in saline (0.25 mg/kg); groups C–E, received MFM at 100, 300 or 1000 mg/kg body weight, respectively. All extracts were re-suspended in saline + 3% DMSO and administered orally, by gavage (1 ml). The test was performed according to Capasso *et al*.[13] Immediately after dosing, the animals were separately placed in cages suitable for collection of the faeces. After 8 h of drug administration, the faeces were collected and weighed. Thereafter, food and water were given to all rats and faecal outputs were weighed after 16 h.

Effect on intestinal motility

The method described by Jansen and Jageneau[14] and Wong and Wai[15] was used to test the effect of MFM on intestinal motility. The animals were divided into five groups ($n = 6$) as follows: group A, negative control received normal saline orally; group B, reference group received bisacodyl in saline + 3% DMSO (0.25 mg/kg); groups C–E received MFM at 100, 300 or 1000 mg/kg body weight, respectively. The animals were previously fasted for 12 h, but were allowed free access to water. All extracts were resuspended in saline + 3% DMSO and administered orally, by gavage (1 ml). After 40 min, 1 ml 10% charcoal suspension in 5% acacia solution was administered to each animal orally. The animals were killed after 20 min and the abdomens were opened. The small intestines were dissected out and placed on a clean surface. The distance travelled by the charcoal meal from the pylorus was measured. The entire length of the small intestine was measured and the percentage distance travelled by the charcoal plug along the small intestine (from the pylorus to the caecum) was estimated for the extract, control and the reference drug.

Acute oral toxicity

The acute oral toxicity was conducted in compliance with Costa-Silva *et al*,[16] with slight modifications. After acclimatization, eight rats were randomly divided into five groups. Group A, control group received the vehicle (saline + 3% DMSO) in a volume of 1 ml/100 g body weight by gavage; groups B–E: received MFM at a dose of 10, 100, 1000 or 2000 mg/kg, respectively.

All animals were observed individually for clinical signs of toxicity immediately and at 1, 2, 4, and 8 h after dosing. Observations were focused on changes in skin, fur, eyes, mucous membranes, respiratory system, autonomic and central nervous systems as well as somatomotor activity and behavioural patterns. The number of animals was noted after 24 h and then maintained for a further 14 days with a once daily observation. Animals were weighed on day 0, and on days 7 and 14. At the end of the study, all surviving animals were killed. Gross pathological examinations of all major internal organs such as heart, lungs, livers, kidneys were performed.

Subchronic toxicity

After acclimatization, eight rats were randomly divided into four groups. Group A, control group to which saline + 3% DMSO were administered orally; groups B–D received a daily dose of MFM at 100, 300 or 1000 mg/kg body weight, respectively, for 42 days. In each case the volume administered was 1 ml/100 g body weight. Each animal was marked and each day for 42 days its body weight, consumption of water and food were measured and behaviour observed.

Biochemical and haematological analyses

At the end of the study, all surviving animals were fasted overnight but with free access to water. Animals were anaesthetized by intraperitoneal injection with pentobarbital sodium (40 mg/kg). Blood samples were collected from the common cardiac puncture and used to measure haematological and biochemical parameters. The haematological parameters assessed included white blood cell count (WBC), differential leucocyte count, red blood cell count (RBC), haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC). The biochemical parameters assessed included glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, globulin, total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglyceride and total cholesterol.[17,18]

Histopathological analysis

On day 42 after blood collection for biological analysis, all the animals were killed and the principal vital organs were removed and macroscopically analysed. After macroscopic analysis, representative fragments of liver and kidneys were subsequently fixed in a 10% solution of buffered formalin

(pH 7.4) and enclosed in paraffin. Five-micrometer sections were obtained and coloured with haematoxylin–eosin for evaluation under an optical microscope.

Statistical analysis

For the in-vivo assays and biochemical analyses, values were presented as means ± standard error of mean (SEM). For the in-vitro antitumoral assays, the results were presented as mean ± standard deviation (SD) for at least two independent experiment performed in duplicate. Statistical differences between the treatments and the controls were tested by one-way analysis of variance followed by the Bonferroni test using the GraphPad Prism 4 statistic computer program. A difference in the mean values of $P < 0.05$ was considered to be statistically significant.

Results and Discussion

In-vitro antitumour activity

The antitumour activity of MFM was evaluated for three lineages of cancer cells: two leukaemic, HL60 (human promyelocytic leukaemia cells) and Jurkat (human T cell lymphoblast-like cell line), and a human breast adenocarcinoma cell line (MCF-7)

MFM (20 μg/ml) was cytotoxic against HL60 and Jurkat cells, with 89 and 83% growth inhibition, respectively. It is important to point out that for both cells the reduction of the cellular proliferation was closer to the reference drug (etoposide) (Figure 1a).

To propose a mechanism of cytotoxic action of MFM, its ability to induce apoptotic death was evaluated (Figure 1b). The method described by Nicoletti *et al.*[12] used here is based on the principle that the apoptotic cells, among other typical features, are characterized by DNA fragmentation and, consequently, loss of nuclear DNA content. Apoptotic nuclei appear in the analysis as a broad hypodiploid DNA peak, which was easily discriminable from the narrow peak of cells with normal (diploid) DNA content. This simple and reproducible method should prove useful for assessing apoptosis of specific cell populations in heterogeneous tissues. The results presented here clearly demonstrated that MFM did not induce an increase of subdiploid DNA content (DNA fragmentation) in HL60 and Jurkat cells when compared with the respective control (DMSO 0.05%).

Thus, MFM induced nonapoptotic cell death, based upon the evidence that it reduced cell viability but it did not induce a much lower level of DNA fragmentation compared with etoposide, the positive control. This suggested that MFM at the tested concentration (20 μg/ml) may have induced another kind of cell death. It is well described that different substances can induce cytotoxicity. Drug sensitivity is likely correlated with the accumulation of apoptotic and nonapoptotic cell deaths, which may influence overall tumour response in anticancer treatment.[19] Nonapoptotic cell death is mainly attributed to autophagy. Death-inducing agents such as tumour necrosis factor (TNF) and staurosporin induced cytotoxicity without producing apoptotic changes. Mechanisms of apoptotic and nonapoptotic cell death induced by anticancer treatment have provided critical information not only for understanding tumour response in terms of signal transduction pathways of cell death, but also for creating an opportunity to design targeting therapy for promoting cell death.[20]

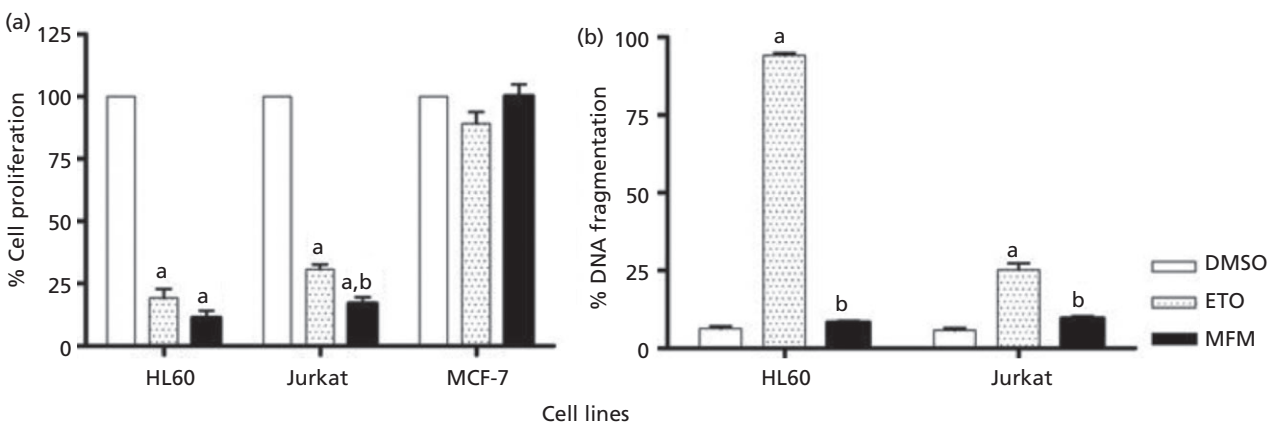

Figure 1 Effect of the methanolic extract of the aerial parts of *Mitracarpus frigidus* on cell proliferation (a) and on DNA content of tumour cell lines (b). HL60, Jurkat and MCF-7 cell lines were incubated with 20 μg/ml methanolic extract of the aerial parts of *M. frigidus* (MFM) or with 0.05% dimethyl sulfoxide (DMSO) or 14 μM etoposide (ETO) for 24 h. Each datum represents mean ± SD for at least two independent experiments performed in duplicate. [a]Statistically different from negative control (DMSO). [b]Statistically different from positive control (ETO) (analysis of variance followed by Bonferroni, $P < 0.05$).

Laxative and intestinal motility effects

The results of the laxative and of the intestinal motility effects caused by MFM are shown in Figure 2.

To evaluate the laxative activity, faeces were examined 8 and 16 h after treatment. In this study, an oral administration of MFM significantly increased the production of faeces 0–8 h after its administration (Figure 2a). The laxative activity of MFM was similar to that of the reference drug, bisacodyl. Importantly, treated animals showed few pasty stools, without however, the appearance of watery stools. Likewise, after 16 h, although all groups increased the amount of faeces produced, no difference in the weight of stools was observed among the different treatments, except for the group treated with saline (Figure 2b). This probably occurred because after 8 h of treatment, animals were allowed free access to food.

Regarding the effect on intestinal motility, the groups treated with MFM showed a significant increase in the pathway of the coal compared with the group treated with saline and bisacodyl (Figure 2c). Furthermore, we observed no relationship with the dose administered. The significant difference between the activity of MFM and the reference drug was probably due to the fact that bisacodyl only began to exert its activity between 6 and 12 h after administration.

Free and anthraquinone *C*- and *O*-glycosides were detected in MFM. The contents of total anthraquinones (total aglycones + total glycosides) and total anthraquinones glycosides were 7.3 ± 0.03 and 5.4 ± 0.05% w/w calculated as emodin, respectively. The laxative effect of anthraquinones is well known, which suggested that these compounds could, in part, be involved in the activity found. The laxative effect is caused by two independent mechanisms, namely changes in colonic motility leading to an accelerated large intestinal transit, and alterations in colonic absorption and secretion resulting in fluid accumulation.[21,22] Both mechanisms are dependent on an interaction of the laxative with the colonic epithelium. Anthraquinone laxatives are used in medicinal practice for diagnostic procedures such as a barium enema, which requires a clean colon, and sometimes for the short-term treatment of constipation.[23]

Toxicological evaluation

Acute toxicity

No toxic symptoms or death were observed in any of the animals and they lived up to 14 days. An autopsy at the end of the experimental period revealed no apparent changes in any

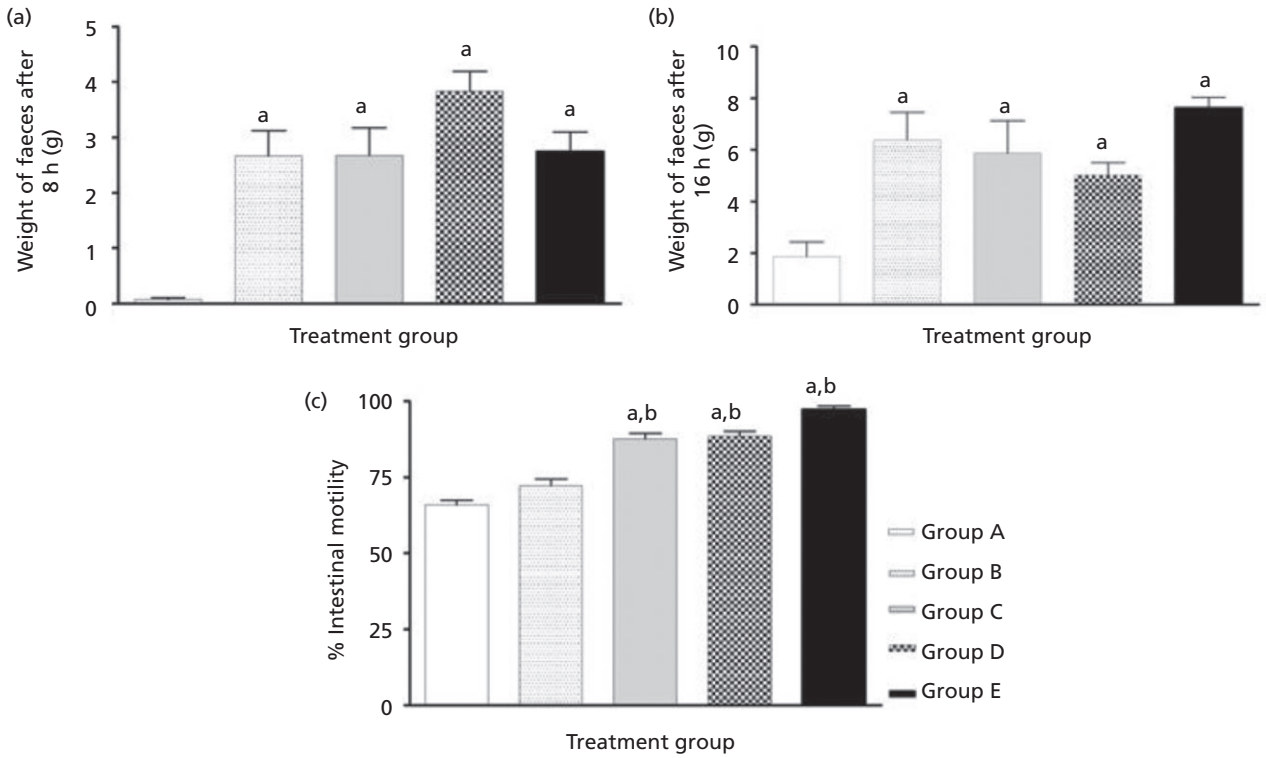

Figure 2 Laxative activity (a, b) and intestinal motility effect (c) of the methanolic extract of the aerial parts of *Mitracarpus frigidus* in rats. Group A, saline; group B, bisacodyl; group C, 100 mg/kg methanolic extract of the aerial parts of *M. frigidus* (MFM); group D, 300 mg/kg MFM; group E, 1000 mg/kg MFM. The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control (group A). [b]Statistically different from positive control (group B) (analysis of variance followed by Bonferroni, $P < 0.05$).

organs. There were no changes either in the corporal weight or in the weight of the principal organs and all animals exhibited a gain in body weight.

Therefore, the minimum lethal dose (LD50) of MFM to male Wistar rats was greater than 2000 mg/kg. This result characterized MFM as being of low toxicity, as described by Larini,[24] which classified oral toxicity as extremely toxic (LD50 less than 25 mg/kg), highly toxic (LD50 between 100 and 500 mg/kg), moderately toxic (LD50 between 500 and 2000 mg/kg) and low toxicity (LD50 above 2000 mg/kg).

Subchronic toxicity

Treatment evolution

The effect of the oral administration of MFM on water consumption, food intake and body weight gain were evaluated daily during subchronic treatment (Figure 3). There was an increase in water consumption in the groups treated with MFM, and this increase was directly proportional to the administered dose (Figure 3a). On the other hand, the groups treated with MFM showed a reduction in food intake (Figure 3b). Figure 3c shows the average body weight gain of animals accumulated every two weeks. A significant decrease in the groups C and D was observed in the first two weeks when compared with the control group. Thereafter, a stabilization of body weight gain was observed for all groups.

Table 1 represents the final weight of the animals and of the principal organs. No significant difference was found when comparing the groups treated with control, except of liver weight in the group treated with 1000 mg/kg MFM.

Biochemical analyses

The biochemical parameters after subchronic administration of MFM at different doses are presented in Table 2. The AST, ALT, ALP and total bilirubin, direct and indirect levels showed no significant changes between the groups treated with MFM and the vehicle. However, in the group treated with 1000 mg/kg MFM there was a significant increase in AST level. AST, ALT, ALP and total direct and indirect bilirubin are important parameters for evaluating hepatoprotective functions and biliary system.[25] The increase in AST level is very nonspecific, since this aminotransferase is abundant in cardiac tissues, skeletal muscles, brain and kidney.[26]

To assess the protein profile analyses on total protein, albumin, globulin and albumin and globulin ratio (A/G) were performed. As shown in Table 2, a significant decrease in total protein and globulin levels of all groups treated with MFM was observed when compared with the vehicle group.

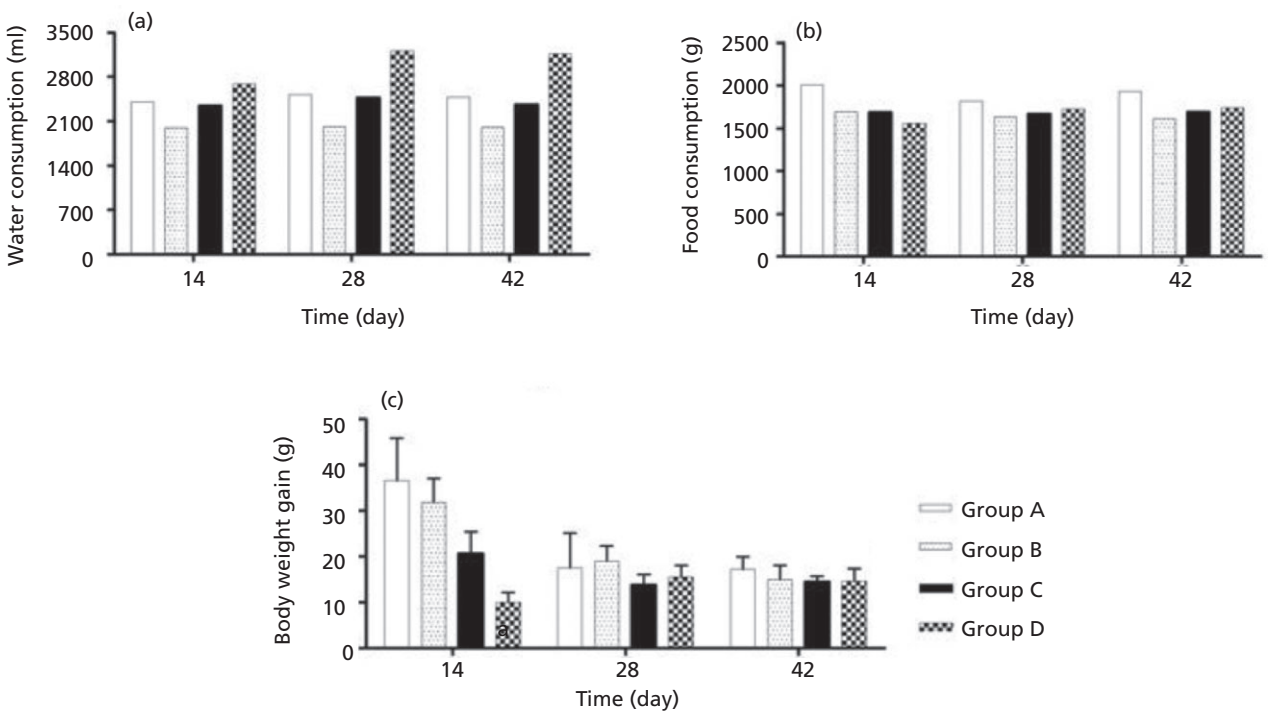

Figure 3 Consumption of water and food, and body weight gain of animals accumulated every two weeks after treatment with methanolic extract of the aerial parts of *Mitracarpus frigidus*. Group A, saline; group B, 100 mg/kg methanolic extract of the aerial parts of *M. frigidus* (MFM); group C, 300 mg/kg MFM; group D, 1000 mg/kg MFM. The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control (group A) (analysis of variance followed by Bonferroni, $P < 0.05$).

Table 1 Body weight of animals and their principal organs after 42 days of treatment with the methanolic extract of the aerial parts of *Mitracarpus frigidus*

Body weight of animals and principal organs	Group A Saline	Group B 100 mg/kg	Group C 300 mg/kg	Group D 1000 mg/kg
Mean weight of animals (g)	310.4 ± 7.4	288.5 ± 8.3	281.8 ± 4.3	285.0 ± 9.0
Heart (g)	1.1 ± 0.04	1.1 ± 0.01	1.2 ± 0.05	1.3 ± 0.08
Liver (g)	9.3 ± 0.4	9.1 ± 0.3	9.5 ± 0.1	10.7 ± 0.2[a]
Right kidney (g)	1.1 ± 0.05	1.2 ± 0.03	1.2 ± 0.02	1.2 ± 0.05
Left kidney (g)	1.1 ± 0.06	1.2 ± 0.03	1.2 ± 0,03	1.3 ± 0.06

The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control group (group A) (analysis of variance followed by Bonferroni, $P < 0.05$).

Table 2 Biochemical and haematological parameters after 42 days of treatment with the methanolic extract of the aerial parts of *Mitracarpus frigidus*

Biochemical and haematological parameters	Group A Saline	Group B 100 mg/kg	Group C 300 mg/kg	Group D 1000 mg/kg
Glucose (mg/dl)	154.8 ± 12.0	142.0 ± 3.5	136.6 ± 6.9	120.2 ± 4.8[a]
Total protein (g/dl)	7.0 ± 0.2	4.9 ± 0.6[a]	5.3 ± 0.6	4.1 ± 0.4[a]
Albumin (g/dl)	3.8 ± 0.3	3.4 ± 0.4	4.0 ± 0.2	4.0 ± 0.2
Globulin (g/dl)	3.2 ± 0.4	1.8 ± 0.5	1.5 ± 0.5[a]	0.8 ± 0.3[a]
A/G	1.2 ± 0.3	1.5 ± 0.3	5.2 ± 2.2[a]	6.5 ± 3.0[a]
ALP (U/l)	34.6 ± 2.4	29.3 ± 7.5	34.8 ± 7.2	25.2 ± 7.2
AST (U/l)	3.8 ± 0.2	3.8 ± 0.3	7.0 ± 2.3	15.7 ± 1.3[a]
ALT (U/l)	16.7 ± 7.4	13.2 ± 1.6	14.9 ± 1.2	24.3 ± 1.0
Total bilirubin (mg/dl)	1.1 ± 0.01	0.7 ± 0.01	0.6 ± 0.01	0.5 ± 0.01
Direct bilirubin (mg/dl)	0.67 ± 0.01	0.3 ± 0.01	0.4 ± 0.02	0.3 ± 0.01
Indirect bilirubin (mg/dl)	0.42 ± 0.01	0.4 ± 0.01	0.2 ± 0.01	0.2 ± 0.02
Urea (mg/dl)	45.7 ± 3.0	39.3 ± 3.4	30.9 ± 5.2	50.8 ± 5.0
Creatinine (mg/dl)	0.7 ± 0.1	1.6 ± 0.6[a]	1.6 ± 0.8[a]	1.3 ± 0.2[a]
Total cholesterol (mg/dl)	92.9 ± 4.9	41.8 ± 9.0[a]	47.2 ± 8.7[a]	51.1 ± 6.3[a]
Triglycerides (mg/dl)	86.7 ± 48.4	116.7 ± 19.1	136.0 ± 10.8	160.0 ± 25.0
RBC (10^6/μl)	5.6 ± 0.2	5.2 ± 0.3	5.1 ± 0.1	4.5 ± 0.3
Haemoglobin (g/dl)	17.0 ± 3.3	21.1 ± 2.3	23.8 ± 0.9	20.7 ± 1.2
Haematocrit (%)	48.4 ± 0.7	41.7 ± 3.2	46.3 ± 0.7	42.3 ± 1.7
MCV (fl)	86.8 ± 0.8	80.6 ± 4.1[a]	90.3 ± 0.8	94.2 ± 4.1
MCHC (g/dl)	35.1 ± 0.8	50.6 ± 4.0[a]	51.4 ± 0.8[a]	48.8 ± 4.0[a]
MCH (pg)	30.4 ± 0.1	40.8 ± 0.1[a]	46.4 ± 0.1[a]	46.0 ± 0.1[a]
WBC (10^3/μl)	3.2 ± 0.4	3.3 ± 0.2	4.2 ± 0.2[a]	4.2 ± 0.2[a]
Basophil (%)	1.0 ± 0.25	1.0 ± 0.01	1.0 ± 0.01	1.0 ± 0.01
Eosinophil (%)	4.8 ± 0.6	3.2 ± 0.5	2.7 ± 0.3[a]	2.8 ± 0.3[a]
Monocyte (%)	16.0 ± 3.6	12.7 ± 2.7	10.5 ± 1.3	9.0 ± 1.5
Neutrophil (%)	42.8 ± 8.6	47.8 ± 4.6	46.0 ± 2.7	49.3 ± 1.3
Lymphocyte (%)	35.3 ± 4.6	35.3 ± 3.4	41.5 ± 3.3	39.5 ± 0.5

The values shown are mean ± SEM ($n = 8$). [a]Statistically different from negative control group (group A) (analysis of variance followed by Bonferroni, $P < 0.05$). A/G, albumin and globulin ratio; ALP, alkaline phosphatase; AST, aspartate aminotransferase: ALT, alanine aminotransferase; RBC, red blood cell count; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; MCH, mean corpuscular haemoglobin; WBC, white blood ell count.

However, these results did not mean any kind of toxicity of MFM, as only the enhancement in the level of serum proteins is an indication of tissue injury and reflection of hepatic toxicity.[27] The histological analyses of the liver revealed no significant change, as shown by a normal lobular architecture and portal-space containing arterioles, venules and bile ducts (Figure 4a and c).

The renal function was assessed by blood urea nitrogen and creatinine.[25] Animals treated with MFM presented a moderate increase in creatinine levels, however this trend was not observed for urea levels (Table 2). The increase observed may have been clinically insignificant as the reference values of creatinine were 0.39–2.29 mg/dl.[28] Urea and creatinine are compounds derived from proteins, which are eliminated

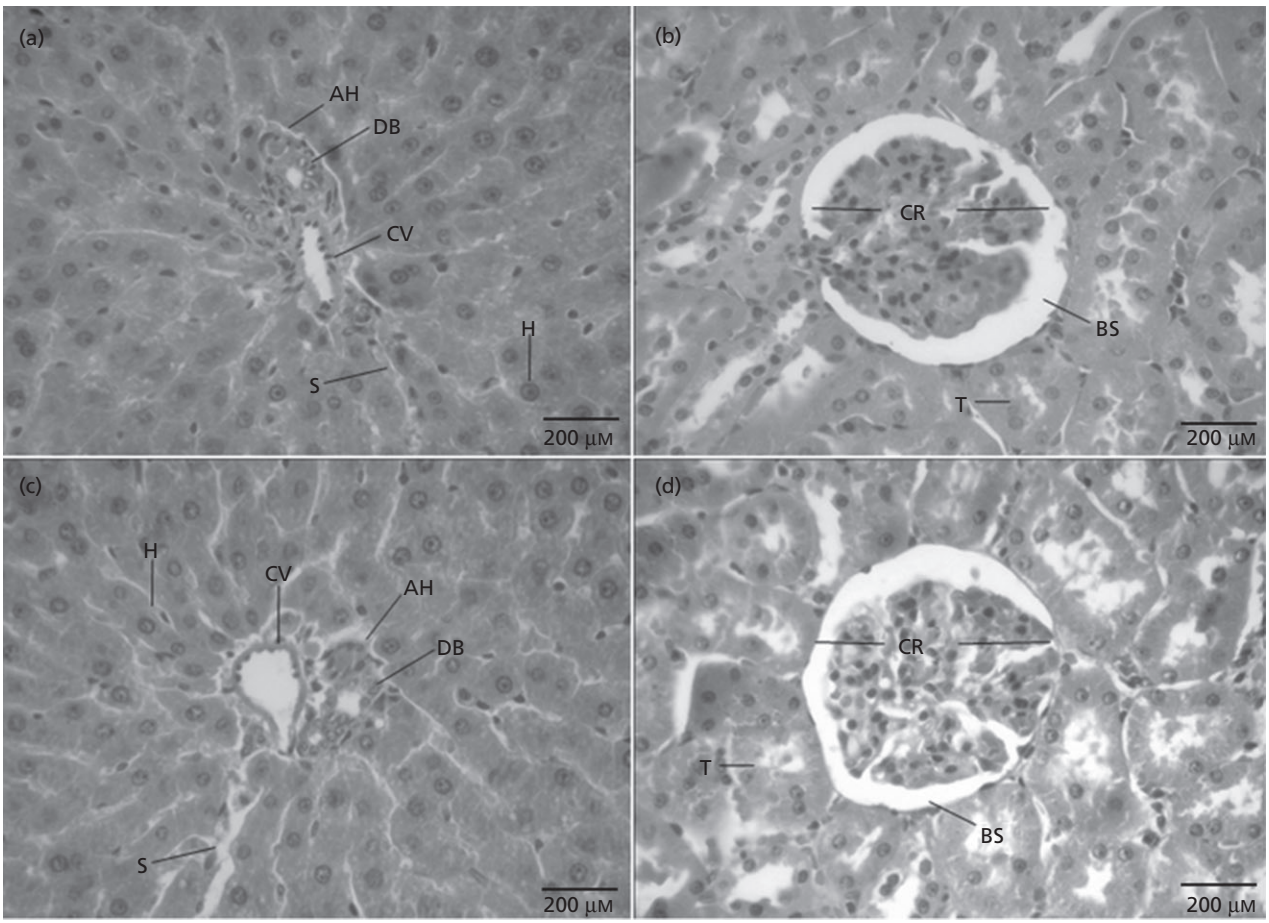

Figure 4 Photomicrographs of the liver and kidney from rats treated with saline (a and b, respectively) or 1000 mg/kg methanolic extract of the aerial parts of *Mitracarpus frigidus* (c and d, respectively) in a subchronic oral toxicity evaluation. Evaluation lasted for 42 days. Cross-sections were stained with haematoxylin and eosin. The liver cross-section shows (at magnification 40x) hepatic artery (AH), bile duct (DB), central vein (CV), sinusoids (S), hepatocytes (H), all clearly conserved. Cross-section of kidney shows (at magnification 40x) renal corpuscles (CR), tubules (T) and Bowman's space (BS), all conserved.

by the kidney. When their blood levels are high, there is nitrogen retention that may provoke renal diseases. Satyanarayana *et al*.[29] reported that renal damage was obtained only when creatinine and urea increased concomitantly. Moreover, in the histological analysis, no alterations in kidney morphology were observed. In all cases, renal cortex and renal corpuscles were preserved, as well as all types of tubules (Figure 4b and d). Finally, MFM was unlikely to affect hepatic and renal function.

Besides these parameters, cholesterol and triglycerides were analysed to determine the serum lipid profile of groups throughout the experiment. Table 2 shows the significant decrease in cholesterol levels in the MFM-treated groups when compared with control group. On the other hand, there was no significant change in triglycerides levels between groups. Cholesterol and triglycerides are parameters associated with coronary artery diseases. Moreover, these parameters are used for evaluation of hyperlipidaemia.[26]

Regarding the values of glucose, a reduction was observed in the group treated with 1000 mg/kg MFM. According to Harkness and Wagner,[30] in rodents glycaemic levels present large physiological fluctuations ranging between 50 and 135 mg/dl, so the glycaemic changes observed were within the reference range and may have been due to physiological fluctuations and not to treatment with MFM.

Haematological analyses

Analysis of blood parameters is relevant to risk evaluation as the changes in the haematological system have a higher predictive value for human toxicity when the data are translated from animal studies.[31] The haematological profile of treated

rats showed no significant difference with the control group, except for WBC and eosinophil which increased and decreased in the groups treated with MFM 300 and 1000 mg/kg, respectively. An increase in WBC directly indicated the strengthening of the organism defence.[32,33] This elevation in total leucocyte count suggested that MFM may have contained biologically active principles that had the ability to boost the immune system.

Conclusions

MFM presented a laxative potential probably due to the presence of free and glycosidic anthraquinones. Moreover, MFM presented cytotoxic activity against leukaemic cells which indicated a potential antitumour activity. Further experiments are necessary to elucidate the cytotoxic mechanism induced by MFM. It is important to point out that this study has provided valuable data on the acute and subchronic oral toxicity profile of *M. frigidus* that should be very useful for any future in-vivo and clinical study of this plant.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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