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GASTROPROTECTIVE, TOXICOLOGICAL AND IMMUNOTOXICOLOGICAL EVALUATION OF *AUSTROPLENCKIA POPULNEA*

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ABSTRACT

Austroplenckia populnea (Celastraceae), is used popularly in Brazil as anti-dysenteric. However, antispermatogenic and gastric protective activity were observed. The objective of the present study was the evaluation of the gastroprotective activity, toxicity and immunotoxicity of the ethanol extract of *A. populnea* in rodents. Doses of 1000 and 2000 mg/kg were utilized in the sub-acute toxicity and immunotoxicological tests. No alterations in the behavior, body weight or consumption of water and ration were detected. A few alterations in the hematological parameters, were observed, such as biochemical ones, which decreased or remained constant, but without clinical significance. There was a discrete variation in the hyper-reactivity of the white splenic pulp in the histopathological evaluation. A modulation of the humoral immune response was observed in the immunotoxicological evaluation, but without interference in the cellular immune response. Considering the results obtained, it is believed that the continuation of the study of this extract is justifiable.

KEYWORDS: ***Austroplenckia populnea*; gastroprotective; toxicity; immunotoxicological; rodents.**

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INTRODUCTION

Austroplenckia populnea (Reiss.) Lund, a tropical species of the Celastraceae family, is present in the Brazilian "cerrado", being popularly known as "Mangabarana", "Mangabeira-Brava" and "Marmelinho do Campo" and it is used in traditional medicine as anti-dysenteric[1]. Mazaro et al.[2] observed an antispermatogenic effect of the hexane extract of *A. populnea*, while Seito et al.[3] demonstrated that methanol and hexane extracts presented gastric protective activity.

Various studies have recently been performed in the attempt to identify natural products with gastric protective activity as a therapeutic alternative, since many of the synthetic products utilized have not been completely effective, in addition to producing many adverse effects[4]. However, the use of natural products is normally compromised by a false concept of security, just because they are "natural". In addition, the fact that the use of medicinal plants is considered to be an alternative to synthetic products and the nonrecognition of these substances as bioactive chemical substances can represent a significant clinical factor, since many of them could be toxic[5].

Immunotoxicity tests have also become the object of studies involving natural products since any xenobiotic that can affect the complex interaction of the immunological system with other systems is considered immunotoxic, although it be recognized that these reactions may be more difficult to identify in pre-clinical studies. Thus, lymphoproliferation assays and determination of the immunoglobulin levels have been utilized to study the phenomenon of individual variation in immunological reactions as a function of the exposure to xenobiotic substances, which are difficult to detect during the pre-clinical evaluation of toxicity[6,7]. The objective of the present study is the evaluation of the gastroprotective effect, toxicity and immunotoxicity of the ethanol extract of *A. populnea* in rodents.

MATERIALS AND METHODS

(i) Botanic material

Austroplenckia populnea (Reiss) Lund leaves were collected during the months of September and November of 2003 at the Serra do Curral, Nova Lima, Minas Gerais, Brazil. The sample was compared with exsiccate number 10.473, available in the Herbarium of the Museum de História Natural of the Universidade Federal de Minas Gerais, Brazil, for confirmation of the authenticity of the species.

(ii) Preparation of the ethanol extract (EE)

Dried and ground *A. populnea* leaves were submitted to continuous extraction with ethanol in a Soxhlet apparatus. The ethanol solution was filtered through a cotton wad in a glass funnel to remove any insoluble residues. The ethanol was completely removed from the filtrate under vacuum on a rotary evaporator at a constant temperature of 50 ºC. The oily, dense residue was transferred to a porcelain capsule and heated at 50 ^{o}C in a forced air hood. After drying, the solid residue was ground in a glass mortar, sifted through a 40-mesh sieve and stored in a cool, dry environment during the analysis period.

(iii) Phytochemical studies

The dry EE from *A. populnea* was tested for the presence of anthracenes, saponins, cardiotonics, flavonoids, polyphenols, tannins, alkaloides and coumarins. The method employed was thin layer chromatography using adequate eluents and specific reagents.

a. Animals

Swiss mice (35 g; 6 to 8 weeks old) and Wistar rats (200 g; 8 to 9 weeks old) from the animal house of the Faculdade de Farmácia of the Universidade Federal de Minas Gerais (UFMG) were used. The animals were acclimatized in the experimental room for five days prior to each experiment and remained under the following controlled conditions

during the experiments: temperature (25 ± 2 °C), humidity (50 to 60%), and a 12-h (07:00 - 19:00 h) light and dark cycle. Food (pellet ration) and water were available *ad libitum* with the exception that the animals were fasted overnight before each experiment, although with free access to water. The experimental protocols were approved by the Ethics Committee on Animal Experimentation (CETEA) of the UFMG (Protocol No. 016/03).

(iv) Evaluation of gastric lesions in rats

Five groups of animals (n = 5) were used. Group 1: control solution (5% Tween 80); Group 2: 180 mg/kg of ethanol extract (EE); Group 3: 360 mg/kg of EE; Group 4: 720 mg/kg of EE; Group 5: 500 mg/kg of sucralfate (Sigma, Brazil). One milliliter of absolute ethanol was administered *p.o*. 60 min after the administration of each of these solutions. The animals were sacrificed 60 min after the application of the ethanol, and the stomachs were removed, opened along the large curvature and immersed in saline solution for preservation.

The lesions were scored with the use of an eight-point scale and by the index of gastric irritation. The lesions were classified on the scale as follows[8]: 0, no lesion; 1, one hemorrhagic lesion shorter than 5 mm and with a width less than 2 mm; 2, one hemorrhagic lesion longer than 5 mm with a width less than 2 mm; 3, more than one hemorrhagic lesion longer than 5 mm with a width less than 2 mm; 4, one hemorrhagic lesion longer than 5 mm and wider than 2 mm; 5, two or three hemorrhagic lesions longer than 5 mm and wider than 2 mm; 6, four or five hemorrhagic lesions longer than 5 mm and wider than 2 mm; 7, over six hemorrhagic lesions longer than 5 mm and wider than 2 mm; 8, complete lesion of the stomach.

The ulceration index was determined by the same analyst and calculated from the sum of the largest diameter of each lesion[9]. The analyst had no knowledge of the treatment received by the animals.

(v) Study of the acute toxicity in mice

Groups of 10 Swiss mice (five males and five females) were used. A 2000-mg/kg dose of EE in a 5% Tween 80 suspension was administered *per os*. The animals were observed at 15, 30, 60, 120, 240 and 360 min. During this period, the animals received no food or water. The animals were then observed twice daily (between 09:00 a.m. and 11:00 a.m. and between 05:00 p.m. and 06:00 p.m.) for morbidity and mortality for 13 days while receiving food and water *"ad libitum"*. The animals were then sacrificed, and macroscopic and microscopic necropsies were performed[10].

(vi)Study of the sub-acute toxicity in rats

Wistar rats were utilized under the same conditions as those described for mice. The animals were divided into three groups of 16 animals each (eight males and eight females), one group being the control and two being used for tests with 1000 mg/kg e 2000 mg/kg doses of EE for 30 days (between 09:00 a.m. and 11:00 a.m).

The consumption of water and ration per box was measured every two days and the animals were weighed weekly for adjustment of the doses. After 30 days of treatment, the animals were sacrificed by decapitation[2], and the blood was collected for hematological and biochemical serum exams, while the organs were removed for weighing and for autopsy by macroscopic and histopathological exams.

(vii) Blood analysis

The hematological analyses were accomplished with the whole blood collected in a heparinized glass funnel and a tube with EDTA anticoagulant. Hemoglobin (HB), hematocrit (HT), red blood cell count (RBC), white blood cell count (WBC) and platelet count (C PLAQ) were the parameters evaluated in an ABC VET® apparatus.

The biochemical analyses were performed on the serum obtained after centrifugation of the tubes, without anticoagulant, at 2500 rpm for 15 min. Standard Analisa® kits and a Biotron® spectrophotometer were utilized for the spectrophotometric readings of the following biochemical parameters: Uric acid (AUR), Albumin (ALB), Alanine aminotransferase (ALT), Cholesterol (COLT), Creatinine (CRE),

Alkaline phosphatase (FAL), Glucose (GLI), Total proteín (PROT) and Urea (URE).

(viii) Immunotoxicity study

- *Preparation of the antigen from the membranes of sheep red blood cells (SRBC)* – The preparation of the SRBC antigen was performed according to the methods of Van Loveren et al.[11] and Temple et al.[6].
- *Protein determination* – The protein concentrations of the SRBC antigen and the EE suspension were determined utilizing the method described by Lowry et al.[12].
- *Immunization of the animals* – The immunization was accomplished according to the method of Temple et al.[6]. The animals were subdivided into six groups (n = 5): group 1 (animals treated with the 5% Tween 80 control solution via *p.o.*); group 2 (animals treated with a 1000 mg/kg% dose of EE via *p.o.*); group 3 (animals treated with a 2000 mg/kg dose of EE via *p.o*); group 4 (animals treated with the 5% Tween 80 control solution via *p.o*. and immunized via i.p. on days 1, 3, 5, 15, 18 and 20 with SRBC at a concentration of 2 x 10^6 cells/mL); group 5 (animals treated with a 1000 mg/kg dose of EE via *p.o.* and immunized via i.p. on days 1, 3, 5, 15, 18 and 20 with SRBC at a concentration of 2 x 10^6 cells/mL); group 6 (animals treated with a 2000 mg/kg dose of EE via *p.o.* and immunized via i.p. on days 1, 3, 5, 15, 18 and 20 with SRBC at a concentration of 2 x 10^6 cells/mL). Three days prior to termination of the immunization, a reinforcement dose of 50 µg/500 µL of soluble SRBC antigen was administered via i.v.
- *Preparation of the cell suspensions.* A single cell suspension was prepared in RPMI 1640 (Gibco, Germany) from each 2/3 spleen. Mononuclear cells were washed three times with RPMI 1640 and re-suspended in the same media supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 UI/mL) and streptomicin (50 pg/mL).

(x) Lymphoproliferative response to cell mitogen

Proliferation of splenocytes in response to the T cell mitogen Concanavalina A (Con A) was evaluated using a microculture assay. Cells were cultured at a density of 2 x 10^5 cells/well for five days; every assay was performed in triplicate, with or without 10 µL of a solution containing 6.76-mg/mL EE and 4 µg/mL Concanavalin A (Con A, Sigma, St. Louis, MO), in a 96-well, flat-bottom microplate at 37 ^oC in a humidified atmosphere containing 5% CO_2. After a 72-h incubation period, cultures were pulse labelled with 1 □ci of tritiated thymidine per well. Eighteen hours later, the cells were collected on filter mats using an automated cell harvester. Tritiated thymidine incorporation into cells was measured with a liquid scintillation counter (Tricarb 2100 tr). The results were expressed as the mean of three determinations minus the average background with cells incubated with RPMI 1640-supplemented medium[13].

(xi) Elisa for measuring the IgG response to SRBC

Serum samples were analyzed for IgG antibody specific for SRBC, as described by Temple et al[6].

(xii) Statistical analysis

The statistical analysis was performed by analysis of variance, followed by the Duncan test utilizing the SigmaStat program to evaluate whether there was a significant difference among the groups. The $p<0.05$ level was considered significant. The nonparamentric Kruskal-Wallis test was used for the analysis of results that did not present a normal distribution.

RESULTS

1. Phytochemical studies

The phytochemical study revealed the presence of saponins, flavonoids, polyphenols, tannins and quaternary alkaloids. The polyphenol content was 21%.

2. Evaluation of gastric lesions in rats

The eight-point scale and the ulceration index of the animals treated with 5% Tween 80, 180 mg/kg, 360 mg/kg and 720 mg/kg doses of EE and a 500 mg/kg dose of sucralfate are presented in Table 1.

Table 1 – ***Effect of ethanolic extract (EE) of Austroplenkia populnea on the gastric lesions long diameter sum (A) and in the index of gastric lesions (B) induced by ethanol 100%; 60 minutes after per os treatment of EE, control solution and sucralfate.***

	EE of *Austroplenkia populnea*				
Treatment (mg/kg)	Control	180	360	720	Sucralfate (500)
A	6.4 ± 0.55[a]	5.4 ± 0.55 [a,b]	3.4 ± 1.14[a,c]	0.4 ± 0.55 [a,b]	3.8 ± 1.10
B	109.2 ± 8.4[a]	72.8 ± 9.0[a,b]	32.8 ± 10.2[a,c]	9.0 ± 13.2[a,b]	47 ± 12.8

Data are expressed as mean ± S.E.M., n = 6 in each group. Duncan test (P < 0,05).
[a] Significant difference compared to sucralfate
[b] Significant difference compared to control, others doses of EE and sucralfate.
[c] Significant difference compared to control and others doses of EE.

3. Study of the acute toxicity in mice

After *p.o.* administration of the 2000 mg/kg dose of EE from *A. populnea,* no death of any animal occurred during the 14 days of observation.

4. Study of the sub-acute toxicity in rats

No relevant alterations in the groups under study were observed in the daily clinical evaluation. There was a difference between the sexes in weight gain and the consumption of food and water (higher in males for all the parameters). The results of the hematological evaluation are presented in Table 2, and the results of the biochemical evaluation in Table 3. The weights of the organs are presented in Table 4.

Table 2 – ***Hematological parameters after per os treatment for 30 days with ethanolic extract (EE) of Austroplenkia populnea or control solution.***

Treatment (mg/kg)	***Sex***	Red blood cells (x10^6 RBC/mL)	Hemoglobin (g/dL)	Hematocrit (%)	Platelets (x 10^6Plaq/mL	White blood cells (x10^3 WBC/mL
Control (Tween 80 5%)	F	6.70±0.34^{aB}	15.50±0.45^{aB}	35.57±1.73^{aB}	761.12±149.35	6.17±0.93^{aB}
	M	7.15±0.58^{bA}	16.65±0.78^{aA}	37.74±3.29^{bA}	774.75±140.04	8.89±2.37^{aA}
EE (1000)	F	6.83±0.27^{aB}	15.75±1.07^{aB}	36.14±1.77^{aB}	834.75±144.14	6.61±1.87^{aA}
	M	7.56±0.26^{aA}	16.56±0.52^{abA}	39.31±1.16^{aA}	876.25±137.02	7.01±1.78^{bA}
EE (2000)	F	6.64±0.23^{aB}	16.07±0.66^{aA}	34.85±1.31^{aB}	807.75±166.56	5.09±0.76^{aA}
	M	7.25±0.21^{bA}	15.89±0.38^{bA}	36.90±1.29^{bA}	864.00±89.35	6.60±1.40^{bA}

Data are expressed as mean ± S.E.M., n = 8 in each group. Minuscule letters refer to comparisons per treatment and capitals per gender, for each parameter. Duncan test (P <0.05).

Table 3 - ***Biochemical parameters afer per os treatment for 30 days with ethanolic extract (EE) of Austroplenkia populnea or control solution.***

Treatment (mg/kg)	Sex	ALT (U/L)	Alkaline Phosphatase (U/L)	Glucose (mg/dL)	Creatinina (mg/dl)	Total Cholesterol (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)	Urea (mg/dL)	Uric Acid (mg/dL)
Control (Tween 80 5%)	F	69.81±9.90^{aA}	62.90±9.58^{aB}	111.06±12.34^{aA}	0.75±0.10^{aA}	75.04±12.18^{aA}	5.66±0.24	4.06±0.33^{aA}	48.93±4.38^{aB}	1.61±0.14^{bA}
	M	75.36±9.59^{aA}	96.01±6.42^{aA}	77.34±9.94^{aB}	0.53±0.11^{bB}	61.85±10.85^{aA}	5.43±0.34	3.69±0.27^{aB}	60.80±9.16^{aA}	1.52±0.28^{aA}
EE (1000)	F	50.82±9.61^{bB}	47.87±9.95^{bB}	94.32±12.10^{bA}	0.84±0.16^{aA}	83.54±20.54^{aA}	5.52±0.50	3.60±0.41^{bA}	47.60±7.82^{aA}	1.48±0.23^{bA}
	M	68.87±7.00^{abA}	79.00±8.50^{bA}	79.22±10.77^{aB}	0.78±0.10^{aA}	66.16±13.03^{aB}	5.50±0.26	3.52±0.29^{aA}	55.36±6.90^{aA}	1.43±0.21^{aA}
EE (2000)	F	72.15±8.88^{aA}	58.68±9.81^{aB}	93.51±11.22^{bA}	0.57±0.14^{bA}	83.29±13.51^{aA}	5.66±0.39	3.92±0.40^{aA}	52.28±8.47^{aB}	1.86±0.24^{aA}
	M	63.80±8.84^{bA}	85.13±8.85^{bA}	77.10±6.05^{aB}	0.58±0.11^{bA}	71.36±13.21^{aA}	5.41±0.23	3.73±0.35^{aB}	61.96±5.44^{aA}	1.52±0.25^{aB}

Data are expressed as mean ± S.E.M., n = 8 in each group. Minuscule letters refer to comparisons per treatment and capitals per gender, for each parameter. Duncan test (P < 0.05). ALT: alanine amino tranferase.

Table 4 – ***Evaluation of body weight and organs weight after per os treatment for 30 days with ethanolic extract (EE) of Austroplenkia populnea or control solution.***

Treatment (mg/kg)	Sex	Body weight initial	Body weight end	Spleen	Heart	Lung	Stomach	Kidneys	Liver	Adrenal
Control (Tween 80 5%)	F	231.8 + 21.3^{aA}	281.9 + 19.2^{aA}	1.00±0.16^{aB}	0.99±0.14^{aB}	1.55±0.11^{aB}	2.07±0.17^{abA}	2.12±0.10^{aB}	7.66±0.76^{bB}	0.09±0.01^{bA}
	M	302.4 + 31.7^{aB}	397.3 + 34.4^{aB}	1.28±0.27^{aA}	1.40±0.12^{bA}	2.20±0.30^{aA}	2.28±0.18^{aA}	3.08±0.24^{bA}	10.91±0.85^{bA}	0.08±0.02^{aA}
EE (1000)	F	232.5 + 19.5^{aA}	277.8 + 26.9^{aA}	1.01±0.11^{aB}	1.02±0.07^{aB}	1.70±0.16^{aB}	2.14±0.19^{aB}	2.36±0.27^{aB}	9.85±1.52^{aB}	0.13±0.03^{aA}
	M	302.3 + 35.4^{aB}	426.3 + 36.6^{aB}	1.35±0.30^{aA}	1.65±0.17^{aA}	2.27±0.45^{aA}	2.37±0.14^{aA}	3.44±0.37^{aA}	13.04±1.32^{aA}	0.10±0.03^{aB}
EE (2000)	F	236.3+ 19.4^{aA}	283.0 + 24.1^{aA}	0.96±0.17^{aA}	0.89±0.14^{aB}	1.79±0.19^{aA}	1.90±0.23^{bB}	2.09±0.28^{aB}	9.00±1.34^{aB}	0.07±0.02^{bA}
	M	297.2 + 29.2^{aB}	415.1 + 61.2^{aB}	1.15±0.21^{aA}	1.40±0.11^{bA}	2.09±0.45^{aA}	2.28±0.25^{aA}	3.21±0.34^{abA}	12.31±1.16^{aA}	0.08±0.02^{aA}

Data are expressed as mean ± S.E.M., n = 8 in each group. Minuscule letters refer to comparisons per treatment and capitals per gender, for each parameter. Duncan test (P < 0.05).

5. Immunotoxicity study

The results of the lymphoproliferation response are presented in Figure 1 and the humoral response (IgG) in Figure 2.

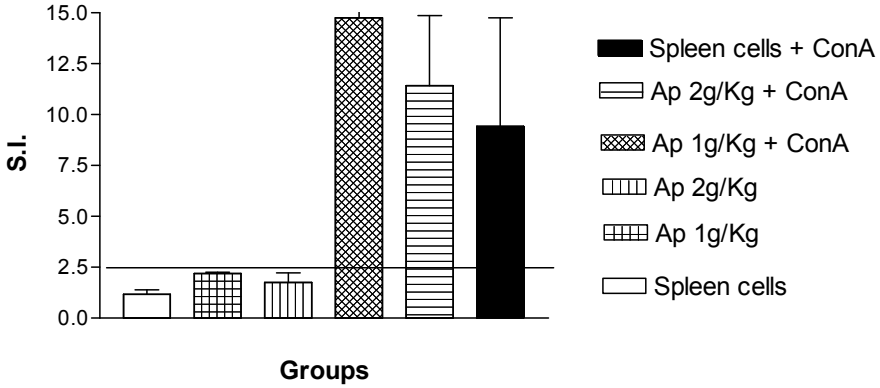

Figure 1 – ***Lymphoproliferation response of spleen cells stimulated by the ethanol extract of A. populnea leaves (1000 mg/kg or 2000 mg/kg) during 30 days of treatment.***

The spleen cells were stimulated with 10 µL of Con A (mitogen) as a positive control and the negative control received no stimulation (n = 5).S.I. = Stimulation index.
Con A = concanavaline A.
Kruskal-Wallis test, * = significantly different (P < 0.05).

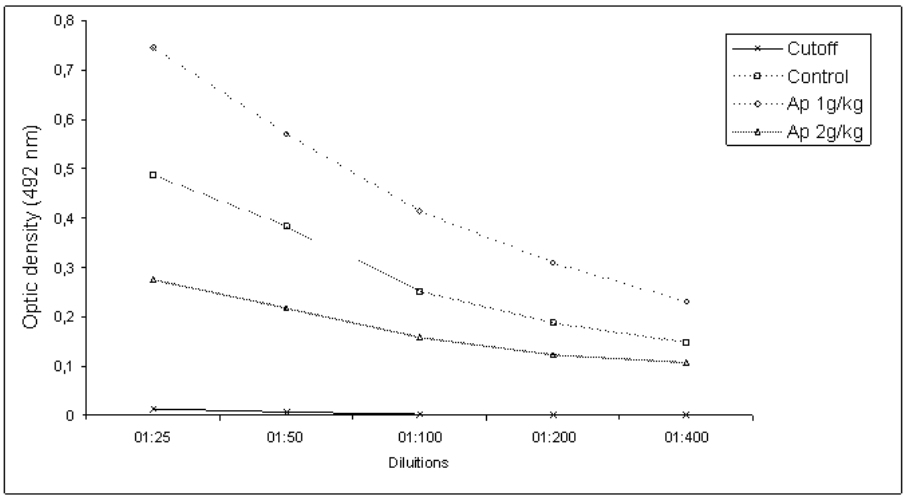

Figure 2 – ***Immunoenzymatic determination of IgG***

Average absorbance at 492 nm obtained in the ELISA reaction performed with diluted serum of animals treated for 30 days with 1000-mg/kg or 2000-mg/kg doses of ethanol extract of A. populnea leaves and untreated (control) animals (n = 5).
Kruskal-Wallis test, * = significantly different (P < 0.05).

DISCUSSION

The presence of flavonoids, tannins and alkaloids has already been described for plants of the *Celastraceae* family[14]. Vieira Filho[15] encountered a significant amount of polyphenols when he studied the ethanol extract of *A. populnea*. However, the presence of quaternary alkaloids in this species is being described for the first time in the present work.

The presence of 21% polyphenols was also observed in this study. These substances could serve as indicators of the quality of the extracts of this species. The high polyphenol content could explain the antiulcerogenic activity observed in the present study. These results are in agreement with those observed by Ariga[16] and Nergard et al.[17] who observed antiulcerogenic activity in species that contained polyphenols. The pathogenesis of the gastric lesion is multifactorial since, when induced by ethanol, depletion of the mucus layer[18], circulatory disturbances in the mucosa[14,19] or, perhaps through direct contact of the necrotic agent with the mucosa cells, the development of free radicals[20] and lipid peroxidation can occur[21].

The results reported here showed that the gastric lesions produced by the administration of ethanol presented the typical aspect of lesions caused by this agent, with focal areas of hyperemia consisting of hemorrhage and edema in bands that descended vertically along the body of the stomach, as reported by La Casa *et al*[8]. These bands occurred principally over the gastric folds, suggesting that alcohol caused primarily local damage where the mucus layer was thinner before being diluted in the gastric secretion[22].

Gastric lesions induced by this model are not inhibited by antisecretory agents, such as cimetidine, but are by agents that increase the mucosa defense factors, such as the prostaglandins[22], or by antioxidants such as tannins and flavonoids[4,21], these compounds being present in the EE of *A. populnea*.

In the present study, the application of a 360 mg/kg dose of EE presented an effectiveness similar to that obtained with 500 mg/kg of sucralfate. The data obtained agree with those presented by Seito *et al.*[3], who observed gastric protection only after administration of a 1000 mg/kg dose of hexane and methanol extracts of *A. populnea* to mice, utilizing the same model. According to these authors, the gastric protection offered by *A. populnea* could be caused by the increase in mucosa protection factors. In fact, the strengthening and/or increase in the mucus layer on the gastric surface may be one of the mechanisms of action of EE, since it was visibly enlarged in the animals treated with this extract.

Thus, the presence of polyphenols, including tannins and flavonoids, in this extract could justify the gastroprotection. Haslam[23] suggested that the role of the polyphenols in the treatment of pathologies could be a result of three general characteristics: their complexation with metal ions (iron, magnesium, vanadium, aluminum, calcium, etc.), their antioxidant properties and ability to capture free radicals and their ability to complex with other molecules, including macromolecules such as proteins and polysaccharides.

The flavonoids can inhibit the proton pump, may increase the liberation of prostaglandins and mucus or may even possess antioxidant activity that can justify their antiulcerogenic action[4,21]. The tannins have an astringent action, which is the capacity to precipitate proteins, combine with them and make them resistant to proteolytic enzymes[23]. The flavonoids probably strengthen the mucus layer[24]. Another possible action of the tannins is their antioxidant activity[21].

According to the World Health Organization[25], the quality control of medicinal plants will determine their effectiveness and safety. The potential of the plants to cause adverse effects is

known. However, there is a great difficulty in determining the frequency of adverse effects because of the precarious pharmacovigilance service in this sector and the lack of reports to the doctors by the patients[26].

The 2000 mg/kg dose of the ethanol extract of *A. populnea* was employed in the acute toxicity test. Since no animal died until the 14th day of observation, this extract was considered to be nonclassified (LD_{50} > 2000 mg/kg)[27].

Toxic effects and obit were not commonly observed in acute experiments with natural products. This feature can be explained by the intrinsic nature of those products, which contain many different compounds, usually in low concentrations. This fact is the opposite of synthetic drugs whose toxicity is more prone to appear in acute assays. The popular perception that natural products do not present toxic effects might be explained within this context, since the recognition of product toxicity is only associated with its use when the effects are manifested immediately after administration[28].

Thus, based on the previous studies of Seito et al.[3] and the results of the present study, in addition to the technical impossibility of preparing and administrating larger doses because doses over 2000 mg/kg were insoluble, it was decided that the doses to be evaluated in the sub-acute toxicological and immunotoxicological studies (30 dias) would be 1000 mg/kg and 2000 mg/kg, administered daily by gavage.

The daily clinical observations are extremely important, as well as the final observations (*end point*)[29]. The doses to be evaluated in the study of the toxicity of repeated doses should be larger than the dose suggested for use in humans. This selection of the dose was a critical point of the study because the loss of 10% of the animals must not occur. In addition, it is important that the maximum tolerable dose suppress body weight gain slightly in repeated dose study (i.e. 10 %) to the survivors, according to the National Toxicology Program (NTP) – United States[29]. The change in body weight is an indicator of adverse side effects[30].

No alterations or differences in behavior of the animals treated with *A. populnea,* compared to the control group, were observed in the daily clinical evaluation. The differences in the body weights of the animals, which are the first indicators of adverse effects of pharmaceutical drugs and chemical products, observed with respect to age and period of exposure, agree with the physiological data for the animals[31].

The consumption of food and water is an important clinical observation for all of the pathways of administration in toxicological studies since adequate nutritional support is indispensable for the physiological maintenance of the animals, which results in a reliable response to the product tested[32]. The consumption of ration and water is different between sexes and during some weeks of the study, but there is no significant alterations among treated and control groups. There are a few alterations among the hematological parameters of the red and white series, but without clinical significance since the values were very close and no histopathological alterations occurred that would sustain statistical differences.

In the study of the biochemical parameters, the serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (FAL) were lower than or equal to those of the control group for both sexes. According to the literature, ALT and FAL are considered markers of the hepatic function[33,34,10]. Therefore, their analysis is of greatest importance since various cases of hepatotoxicity have recently been reported with the use of natural products[35].

In addition to ALT and FAL, the monitoring of the serum lipids is important for evaluation of possible hepatic damage[36]. In the present study, the alterations in the levels of total serum cholesterol relative to that of the control

group were not statistically significant for either of doses employed in the treatment.

An increase in the liver weight relative to that of the control group was observed for both sexes. Hepatomegalia is a clinical aspect already described by Larrey[37] in the use of phytotherapic substances that contain pyrrolizidine alkaloids and by Stickel & Seitz[38] in the use of confrey. However, the decrease or maintenance of the levels of ALT, FAL and total cholesterol and the lack of cellular lesions in the histopatological evaluation of the liver indicate that no hepatic damage occurred in the use of *A. populnea* under the conditions of this study.

With regard to the evaluation of the renal function, there exist various reports of nephrotoxicity with the use of phytotherapic substances[39]. A significant difference in the serum levels of uric acid was detected only among the females that received 2000 mg/kg of the product. And no significant difference was observed between the treated and control groups with respect to the concentration of urea. A statistically significant difference in the level of creatinine relative to that of the control group was also detected in the males that received 1000 mg/kg doses of *A. populnea*. These alterations were not considered significant since the values of creatinine and uric acid for the treated and control groups are very close. Reference values are utilized when no explanations exist for statistically significant differences between the values obtained for treated animals relative to those of the control group [34]. The serum levels of creatinine, uric acid and urea, which are important indicators of renal function, together with the histopathological evaluation of the kidneys, in which no alteration of the tissue was detected, suggest that, in principle, no nephrotoxicity exists. This result is in agreement with those reported by Henry[33].

The levels of glucose were lower in the females treated with *A. populnea* than those of the females of the control group. However, these values are within the normal limits, so they have no clinical significance. No statistically significant differences in the total protein and albumin levels were observed among the groups.

Other significant alterations occurred in the weight of some organs such as the suprarenal bodies of females treated with a 1000 mg/kg dose and the heart and kidneys of males treated with the same dose. However, there was no correlation with the histopathological analysis in which no tissue lesion was detected. The only significant variation observed in the histopathological evaluation was a discrete hyper-reactivity of the white splenic pulp with lymphoid follicles presenting a prominent germinative center. .

An absence of concordance between the studies in humans and the pre-clinical studies exists because of the lack of immunological, laboratorial and even clinical studies. Immunotoxicology is considered to be a bridge between experimental studies and human risk studies[40]. Immunotoxicity studies are performed via the lymphoproliferative response (cellular response) and the determination of specific antibodies (humoral response). These reactions are suggested by the FDA and NTP[7,41], mainly because they possess predictive value for human toxicity, which is hard to extrapolate by the methods previously utilized[42].

The immunotoxicological studies with repeated doses in rodents are regulated by the OECD 407 (studies with exposure for 28 days) and OECD 420 (considers 14 day studies) [43,44]. Both studies should be performed with the maximum dose of 2000 mg/kg[13]. Therefore, the present immunotoxicological study of *A. populnea* was accomplished parallel to the toxicological study using 1000-mg/kg and 2000-mg/kg doses of ethanol extract during 30 days.

The results of the lymphproliferation response are presented in Figure 1, where it can be seen that the EE of *A. populnea* does not stimulate the proliferation of spleen cells relative to the negative control

group. No alterations relative to the positive group were observed in the groups treated with *A. populnea* and whose cells were stimulated with Concanavaline A. Therefore, the EE of *A. populnea* did not interfere in the cellular immunological response.

The immunoenzimatic determination of the IgG anti-SRBC levels is amply described in the literature[6,11,45]. The results encountered after immunization of the animals with SRBC and administrations of the EE of A. populnea for 30 days are presented in Figure 2.

The IgG levels were higher, in comparison with the control group, for the group of animals that received the 1000 mg/kg dose and lower for that which received the 2000 mg/kg dose of *A. populnea.* The use of this product in doses three-fold greater than the effective dose suggested in this work (360 mg/kg) for a prolonged period may lead to increase in the levels of these antibodies. On the other hand, the administration of the 2000 mg/kg dose, approximately six-fold greater than the suggested therapeutic dose, lead to a decrease in this response.

Various studies with other plant species have reported immunological modulation as a result of the administration of natural products, such as the study of Féres[28], who encountered a reduction in the IgG levels in rats treated for 30 days with a 2000-mg/kg dose when he evaluated the immunotoxicological potential of *D. mollis.* Similar results for reduction of the humeral immune response were also obtained by Benencia et al.[46], who detected a dose-dependant immunosupressor activity of *Trichilia glabra,* employed because it presented antiviral, anti-inflammatory and antirheumatic properties, and Alvarez et al.[13], who also encountered a reduced humeral immune response with an increase in the dose when they evaluated the immunotoxic potential of Ochratoxina A (OTA) from a species of *Aspergillus.* Fidler et al.[47] reported an immunosuppressive activity in mice when *Tripterygium wilfordii*, a plant that belongs to the same family is *A. populnea*, was used for the treatment of autoimmune diseases.

CONCLUSION

In conclusion *A. populnea* presented: 1) efficiency in the protection of the gastric mucosa, with the observation of a strengthening and/or in the increase in the mucus layer of the gastric surface; 2) dependability in the acute and sub-acute toxicity studies; and 3) no interference in the immunological response when the effective dose suggested in the present study (360 mg/kg) was used; it is believed that the results obtained justify the performance of sub-chronic tests (90 days) in rodents in a continuation of this study. Such in the evaluation over a prolonged period is important because the adequate antiulcerogenic activity in doses approximately three- and six-fold greater than the effective antiulcerogenic dose (360 mg/kg), presented effects suggestive of an adverse event such as the modulation of the humeral immune response. On the other hand, directed immunotoxicological assays should also be performed since the alterations of this response could suggest possible immunotoxic effects.

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