Evaluation of the immunotoxicological effects of *Dimorphandra mollis* Benth., Fabaceae, in rats

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RESUMO: “Avaliação dos efeitos imunotoxicológicos de *Dimorphandra mollis* Benth., Fabaceae, em ratos”. A *Dimorphandra mollis* Benth., Fabaceae, conhecida como faveira ou fava-d’anta, é uma planta comum do cerrado central do Brasil, muito utilizada por suas propriedades antioxidante, antiplaquetária e, principalmente, como vasoprotetora. Seu principal marcador é a rutina. Este estudo teve como objetivo avaliar a segurança da utilização do extrato seco de *D. mollis* em roedores. O extrato foi extrai...
Benth., Fabaceae, collected in Dimorphandra mollis. DMDE was prepared from the fruits of the plant.

Preparation of DMDE

DMDE was prepared from the fruits of Dimorphandra mollis Benth., Fabaceae, collected in February, 2003, in the woodsy meadow region in the north of the state of Minas Gerais, Brasil. Botanical investigation was performed after herborization of the material and morphological comparison to exsiccate stored in the SAT-CETEC (HX-BH) herbarium. The DMDE development was performed by Planta Medicinal Indústria e Comércio Ltda. After collection of the plant material, the samples were dried at room temperature, ground into powder in knife mills, and submitted to extraction with a mixture of hydro-alcoholic solvents. The concentration of the liquid extract was performed to obtain the dried extract, which had the form of an amorphous, yellow powder. Flavonoid determination in DMDE was performed in triplicate by high performance liquid chromatography (HPLC) using the external standard method (rutin). A reverse phase column RP-18 was employed, with gradient elution by a mixture of solvents, at a temperature of 40 °C, flow rate of 1.0 mL/min and UV detection. This extract was standardized to 76% rutin.

The experimental suspension was prepared daily by dissolving 20% DMDE and 0.05% carboxymethylcellulose (CMC, suspending agent) in 79.95% distilled water. For the control group, a suspension containing 0.05% CMC and 99.95% of purified water was used.

Animals

Eight-week-old female Wistar rats (200±20 g) were furnished by the bioterium of the Faculdade de Farmácia of the Universidade Federal de Minas Gerais. The animals were kept in cabinets for five days before the experiment, to allow acclimatization and from then on, under controlled conditions of temperature (25±2 °C), humidity (50-60%) and 12 h light/darkness (7:00-19:00 h). The animals were fed with food in pellets and water ad libidum, exception for overnight before each experiment and 4 h after the administration of the drugs, although they continued to have free access to water. The experimental protocols were approved by the Ethics Committee on Animal Experimentation (CETEA) of the UFMG (protocol n° 17/2003).

Membrane antigen from sheep red blood cells (SRBC)

The SRBC antigen were determined by the method described by Temple et al., 1993; Van Loveren et al.,1991.

Protein determination

The protein concentrations of the SRBC antigen and the DMDE suspension were determined by the method described by Lowry et al. (1951).

Immunization of the animals

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The rats were immunized intraperitoneally (i.p.) with 2x 106 SRBC on days 1, 3, 5, 15, 18 and 20. Three days prior to sacrificing the animals, a reinforcement dose of 50 µg/500 µL of SRBC soluble antigen was administered intravenously (i.v.) (Ladies et al., 1995; Temple et al., 1993; Van Loveren et al., 1991). The oral administration (p.o.) of DMDE or the control by gavage was performed daily during thirty days. The rats were divided into six groups of five animals. Group 1: animals treated with the control solution, CMC, via p.o.; Group 2: animals treated with DMDE, 1000 mg/kg dose, via p.o.; Group 3: animals treated with DMDE, 2000 mg/kg dose, via p.o.; Group 4: animals treated with the control solution, CMC, via p.o., and immunized via i.p. with SRBC; Group 5: animals treated with a 1000 mg/kg dose DMDE, via p.o. and immunized via i.p. with SRBC; Group 6: animals treated with a 2000 mg/kg dose of DMDE, via p.o., and immunized via i.p. with SRBC.

After thirty days, serum samples from these animals were collected, and the animals were sacrificed by decapitation. After aseptic removal, each spleen was evaluated macroscopically, and approximately one-third of each spleen was separated for histological studies. The remaining two-thirds were used for obtaining the mononuclear cells used in the lymphproliferative response trial.

Clinical laboratory evaluation

Biochemical analyses were performed on the animals’ serum utilizing Kits Analisa and a Biotron spectrophotometer. Total protein, albumin and globulin were determined. The hematological parameters - number of erythrocytes, leukocytes, hemoglobin concentration and hematocrit - were measured on a Device ABC Vet. The differential leukocytes count was also performed.

Pathological evaluations

Representative samples of the spleens were collected from the rats. No histological evaluation was performed on the thymus because the evaluation would have to be very carefully interpreted because of the inherent variability associated with its removal, the majority of the thymuses being partially or totally destroyed during the decapitation. All spleens were fixed in 10% neutral buffered formalin. Processed tissues were embedded in paraffin, cut at a thickness of 5 mm and stained with haematoxylin and eosin. Tissues collected from rats in all groups were processed to slides and examined microscopically.

Preparation of cell suspension

A single cell suspension was prepared from each 2/3 spleen in RPMI 1640 (Gibco, Germany). After aseptic removal of the spleens, they were ground and the mononuclear cells were filtered through sterile nylon. The cells were washed with Fazeaks St Groth saline solution at 1400 rpm during 10 min at 18 °C. The erythrocytes were lysed with ammonium chloride solution, and the cells were washed three times with RPMI 1640 and resuspended in the same medium supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/mL) and streptomycin (50 µg/mL).

Lymphproliferative response

Cells were cultured in triplicate at a density of 2x 105 cells/well in a 96 well flat-bottomed microplate at 37 °C in a humidified 5% CO₂ atmosphere for five days, with or without DMDE. The DMDE concentration was 35 µg/mL and that of concanavalin A (Con A, Sigma, St. Louis, MO) was 4 µg/mL. After incubating for 72 h, cultures were pulsed with 1 µCi of tritiated thymidine per well. Eighteen hours later, cells were collected onto filter mats using an automated cell harvester, and the incorporation of tritiated thymidine into the cells was measured by a liquid scintillation counter (Tricarb 2100, USA). The results were expressed as the mean of three determinations minus the background using cells incubated with RPMI 1640-supplemented medium (Alvarez et al., 2004; Benencia et al., 2000; Peijie et al., 2003; Tulinska et al., 2000).

Elisa measurement of the IgG response to SRBC

The ELISA reaction was performed in polystyrene microplates (Hemobag) sensibilized with 2 µg/100µL/well of the antigen preparation (SRBC). After developing, the absorbancies were read at 492 nm with an automatic reader for microplates (Arriba et al., 2002; Ladics et al., 1995; Temple et al., 1993; Van Loveren et al., 1991).

Statistical analyses

The statistical analysis was achieved with a parametric test for analysis of variance, followed by the Duncan test using the SAEG program. The level of significance used was 5%. Mathematical adjustments were applied to some parameters to make the variable a parametric response. A Kruskal-Wallis non-parametric test was applied for the analysis of results that did not present a normal distribution and that could not be parametricized.

RESULTS

Biochemical and hematological analyses

No significant difference (p>0.05) was observed between the control groups and those treated with the two doses of DMDE or the animals immunized with SRBC in the evaluation of serum proteins (total proteins, albumins and globulins) (Figure 1). Among the hematological...
parameters (Figure 2), those values encountered for erythrocytes, hemoglobin, hematocrit and overall total leukocytes in the rats that received DMDE and/or SRBC are similar to those obtained for the control group ($p>0.05$). When the differential leukocytes count was analyzed, an increase in the number of eosinophiles was observed when a 2000 mg/kg dose of DMDE was used, including the association of DMDE with SRBC. No alteration in the percentages of the other leukocytes occurred.

**Production of antibodies**

No difference between the levels of IgG anti-SRBC (Figure 3) ($p>0.05$) in the control groups and the group that received a 1000 mg/kg dose of DMDE was observed. The levels of IgG were lower in the group that received 2000 mg/kg ($p<0.05$) than those of the control group.

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**Figure 1.** Evaluation of the serum proteins: Total proteins (1.a), Albumin (1.b), globulin (1.c), after treatment for thirty days with a control suspension of carboxymethylcellulose (CMC) and with 1000 mg/kg and 2000 mg/kg doses of the semi-purified dried extract from *Dimorphandra mollis* Benth., Fabaceae (DMDE), with and without the association with sheep red blood cells (SRBC).
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**Lymphproliferative response**

No significant difference ($p<0.05$) in the cells of rats that received 1000 mg/kg and 2000 mg/kg doses for 30 days relative to the control group composed of untreated animas was observed upon stimulation with Con A (Figure 4).

**Figure 2.** Hematological evaluation: Red blood cells (RBC) (2.a), White blood cells (WBC) (2.b), Hemoglobin (2.c), Hematocrit (HCT) (2.d) and Differential leukocyte (neutrophils-2.e, bastons-2.f, cosinophils-2.g, lymphocytes-2.h and monocytes-2.i) after treatment for thirty days with a control suspension (carboxymethylcellulose -CMC) and with 1000 mg/kg and 2000 mg/kg doses of dried extract from *Dimorphandra mollis* Benth., Fabaceae (DMDE) with or without the association with SRBC; $n = 5$; *values above the maximum value were considered to be within the minimum to maximum range of the reference values.
DISCUSSION

The possibility of the development of a pathological condition such as immunostimulation, hypersensibility and immunosuppression that lead to an increase in the susceptibility for the development of tumors and infections is very important, especially in post-transplant patients that received prolonged immunosuppressive therapy (Herzyk & Gore, 2004). The evaluation of different branches of immune response that permit the precise identification of some of these pathological conditions during the administration of an experimental drug is equally important. Some effects induced by drugs can lead to an alteration in the processing and in the presentation of antigens in the synthesis and the functioning of interleukin, in addition to cell differentiation and proliferation (Luster et al., 1992). The European guidelines recommended for immunotoxicology studies with repeated doses in rodents are regulated by the OECD 474 (28 days), and the studies must be conducted with the dose limit of 2000 mg/kg (Alvarez et al., 2004). This immunotoxicological study of D. mollis was conducted with doses of 1000 and 2000 mg/kg for thirty days. The doses used in this study were obtained from LD50 test results (Féres et al, 2006). It is known that a yield of 76.0±3% of rutin is obtained from the DMDE obtained from D. mollis fruits (Féres et al, 2006). The fruits of this species represent an important therapeutic source, especially as a venous tonic and antioxidant, providing an alternative for the prevention and treatment of circulatory problems. A pre-clinical toxicity study demonstrated to be low for this extract (Féres et al, 2006). No alterations in the levels of total protein, albumin and globulin were observed in any of the groups evaluated (Figure 1). This fact indicates that there was probably no liver damage as a result of the administration of DMDE.

In the study of the hematological parameters (Figure 2), the values for red blood cells, hemoglobin, hematocrit and overall white blood cell count were observed to agree with those described by Jain (1986). These results suggest that a 1000 mg/kg dose of DMDE did not promote a change in the hematological parameters, but, when a dose of 2000 mg/kg was used, an increase in the number of eosinophils may suggest the occurrence of hypersensitivity. Although the indication of immunotoxicity may not have clinical relevance, it suggests that its use at this dosage or in repeated doses should be investigated further. The levels of antibodies were evaluated after the stimulation of the immune system with SRBC and the administration of DMDE for thirty days (Figure 3). Experiments on immunization of animals with a suspension of intact sheep red blood cells in a standardized concentration are widely described in the literature (Delaney et al., 2001; Ladics et al., 1995, Temple et al., 1993; Van Loveren et al., 1991), and some researchers have already used the suspension of industrialized SRBC (BioMérieux®) (Alvarez et al., 2004).

No change in the levels of IgG in the group that received 1000 mg/kg of DMDE was observed, and, since this immunoglobulin is a marker for the chronic phase, this result has clinical significance. Therefore, the use of this phytotherapeutic drug at doses approximately twenty times greater than the usual therapeutic dose (15 to 100 mg/kg) for a prolonged period did not alter the levels of specific IgG. However, a decrease in the levels of IgG, as compared to the control and 1000 mg/kg group, was observed for the group that received 2000 mg/kg of DMDE.

Similar results of dose-dependent impaired humoral immune response were obtained by Alvarez et al. (2004), who observed that the humoral immune response decreased with the increase in dose when they assessed the immunotoxic potential of ochratoxina A (OTA) from a species of Aspergillus. The decrease in the levels of antibodies (IgG) observed in this study with the highest dose of DMDE is related to the results found in the histopathological evaluation of the spleen, in which hyperplasia of the white stroma was detected, a result that indicates that this organ reacts to the compound (data not shown).

In accord with the results presented for the lymphoproliferative response (Figure 4), DMDE did not inhibit the lymphoproliferative capacity mediated by the mitogen, thus maintaining the complete capacity for activation, differentiation and proliferation of spleen cells. These characteristics are vital to the effector viability of cell immunity. Therefore, DMDE presented no inhibitory activity on cellular immunity in this study.

In this context, it became necessary to evaluate, through standardized, safe methods, the potential for adverse effects to affect the dynamic and complex immune system. The evaluation of the immune response is indicated, mainly by the FDA (2002) and NTP (Luster et al, 1994), because of its higher predictive value for human toxicity (Olson et al., 2000). The possible immunotoxicological effects identified during the period of exposure to a particular drug are very important because they will be part of the assessment of its risk for use (Descotes, 2004; Olson et al., 2000).

No Immunotoxicological study involving the D. mollis extract has been described in the literature. Studies with other plant species have been reported, such as Benencia et al. (2000), who identified dose-dependent immunosuppressive activity in an extract from Trichilia glabra, which displays anti-viral, anti-inflammatory and anti-rheumatic properties. Sharma et al. (1994) detected an immunostimulatory potential in extracts from leaves of Picrorhiza kurroa, used for
anti-inflammatory, anti-arthritis, gastrointestinal and urinary disorders. Tiwari et al. (2004) also detected immunostimulatory activity in an aqueous extract of *Tridax procumbens* (used for its anti-inflammatory, antimicrobial and hepatoprotective properties).

The results showed that the DMDE did not interfere with the cellular response, but high doses, such as 2000 mg/kg, a dose many times higher than the usual dose, can lead to an increase in the number of eosinophils, suggesting the occurrence of hypersensitivity, and can also cause a reduction in the levels of anti-SRBC antibodies. Therefore, considering the results of this work that showed no immunotoxic action for DMDE up to the dose of 1000 mg/kg, and possible modulation at higher doses such as 2000 mg/kg, the continuation of immunotoxicological studies to ensure the safety and effectiveness of products produced with the flavonoid rutin is considered to be fundamental.

**Figure 3.** Immunoenzymatic determination of IgG anti SRBC. Mean absorbance at 492 nm obtained in the ELISA anti-IgG reaction performed with dilutions of the serum from the animals (n = 5 per group) treated for thirty days with 1000 mg/kg and 2000 mg/kg doses of the semi-purified dried extract from *Dimorphandra mollis* Benth., Fabaceae (DMDE) and immunized with SRBC. Serum dilutions: 1 = 1:25, 2 = 1:50, 3 = 1:100, 4 = 1:200, 5 = 1:400. Duncan test, *Significant difference (p<0.05). - - - cut off*

**Figure 4.** Lymphproliferative response of spleen cells induced by concanavalin A. The vertical bar represents the arithmetic mean for each group. The spleen cells were stimulated with concanavalin A (mitogen) as a positive control for the lymphocyte proliferation tests. The horizontal bar represents the cut-off point. Kruskal-Wallis test for groups with concanavalin A (p<0.05) and Duncan test for the remaining groups (p<0.05). S.I. = Stimulation index.


